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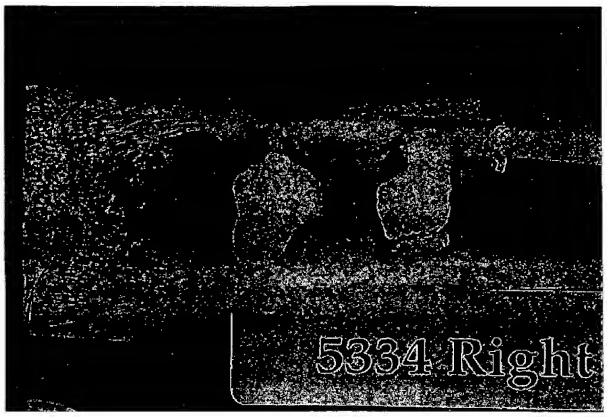

(57) Abstract: A bone precursor composition comprising a cement mixture and a pore-forming agent is provided for bone implant. Preferably, the pore-forming agent has a particle size of 20-500 μm. More preferably, the proportion of the pore-forming agent is 7-40% (w/w). The composition may further include a bioactive agent, preferably a bone morphogenic protein or nucleic acid encoding BMP encapsulated in the pore-forming agent. The moldability of the composition can be modulated by the addition of a binder. The invention provides a kit and implant device comprising the bone precursor composition. The invention also provides an implantable prosthetic device comprising a prosthetic implant having a surface region and a bone precursor material disposed on the surface region. The kit and devices may further comprise one or more additional components including a bioactive agent and a binder. Methods of inducing bone formation and delivering the bioactive agent are provided.

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PORE-FORMING AGENTS FOR ORTHOPEDIC CEMENTS

BACKGROUND OF THE INVENTION

[0001] Bone tissue in the human body comprises the largest proportion of the body's connective tissue mass. However, unlike other connective tissues, its matrix consists of physiologically mineralized, tiny crystallites of a basic, carbonate-containing calcium phosphate called hydroxyapatite distributed in an organized collagen structure. Repair of this tissue is a complex process involving a number of complex cellular functions directed towards the formation of a scaffold and mineralization of the defect followed by an eventual remodeling of the defect site to attain the original structure.

[0002] In a majority of situations, calcium phosphate based implants have been found to be compatible and conducive to bone repair. Hydroxyapatite (HA) has a formula of $Ca_{10}(PO_4)_6(OH)_2$ and the compound is similar in stoichiometric composition to bone mineral and to tooth enamel. Porous hydroxyapatite blocks and particulates have been widely used as an implant to provide structural support as the material is osteoconductive and supports the ingrowth and attachment of bone. However, these hydroxyapatite materials have inconvenient handling properties for most orthopedic procedures (Parsons *et*

al., Annals of the New York Academy of Sciences, 523, pp. 190-207 (1988)). Various calcium cement formulations (i.e. hydroxyapatite cements) have been developed to have improved handling characteristics *in situ* (Tay *et al.*, The Orthopedic Clinics of North America, 30, pp. 615-623). These formulations, however, have deficiencies in that upon implantation, they do not undergo significant resorption.

[0003] To improve the resorption of the HA cement, pore-forming agents such as calcium sulfate have been added to the cement. See, e.g., Parsons *et al.*, *supra*. Subsequent resorption of the pore-forming agent provides a porous HA cement, which allows osteolytic cells to infiltrate the pores and facilitate bone growth. However, inappropriate porosity and pore size of the porous HA cement can interfere with the bone integration process.

[0004] Calcium cements which have a large pore size and high porosity result in excessive resorption rates and weak physical strength of the material, thus, preventing the matrix from providing a scaffold for the newly synthesized bone. In addition, when the rate of bone resorption is faster than the rate of bone growth, an inflammatory response is often observed. Calcium cements that have a small pore size and low porosity result in low resorption rates, which causes encapsulation of matrix particles in the newly formed bone.

[0005] Thus, it would be desirable to develop a cement formulation comprising a bone precursor composition and a pore-forming agent which allows significant resorption, maintains structural integrity in physiological environments, and enables manipulation of the cement *in situ*.

SUMMARY OF THE INVENTION

[0006] The present invention solves these problems by identifying a bone precursor composition comprising a cement mixture and a pore-forming agent. The invention also provides a composition comprising a solid cement and a pore-forming agent. Preferably, the particle size of the pore-forming agent is 20-500 μm, more preferably 20-140 μm, and most preferably, 75-140 μm. In a preferred embodiment, the proportion of pore-forming agent is 10-70% by volume, more preferably, 40-60% by volume. In a preferred embodiment, the proportion of pore-forming is 7-40% by weight, more preferably, 7-25% by weight. In a more preferred embodiment, the proportion of PLGA is 7-14% by weight. The composition of this invention improves the penetration of cellular factors into the cement, while maintaining the physical strength and the handling properties of the implant, thereby improving the regeneration of bone tissue in a living body.

[0007] The invention also provides a composition comprising the bone precursor composition and a bioactive agent such as a bone morphogenic protein (BMP) or a nucleic acid molecule comprising a sequence encoding a BMP. In a preferred embodiment, the bioactive agent is encapsulated in the pore-forming agent. The bone precursor composition or bone precursor composition/bioactive agent mixture can also be used in conjunction with binders to modulate the moldability of the composition at the implant site. The invention also provides a kit comprising the bone precursor composition, and at least one or more additional components including a bioactive agent and a binder.

[0008] In another aspect, the invention also provides an implantable device comprising the bone precursor

composition material, and optionally comprising one or more additional components including a bioactive agent such as a BMP or a binder. The invention also provides an implantable prosthetic device comprising the bone precursor composition and optionally comprising one or more additional components including a bioactive agent such as a BMP or a binder.

[0009] The invention provides a method of inducing bone formation in a mammal comprising the step of implanting in the defect site a composition comprising the bone precursor composition and optionally a binder and/or a bioactive agent. The invention also provides a method of delivering a bioactive agent at a site requiring bone formation comprising implanting at the defect site of a mammal a composition comprising the bone precursor composition and a bioactive agent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1. Gross Image (top) and Faxitron Image (bottom) of right tibia of Animal 5334 at four weeks. From the left, the proximal site, middle site and distal site contain Formulations 1, 2 and 3, respectively.

[0011] Figure 2. Gross Image (top) and Faxitron Image (bottom) of left tibia of Animal 5329 at four weeks. From the left, the proximal site, middle site and distal site contain Formulations 6, 7 and 8, respectively.

[0012] Figure 3. Gross Image (top) and Faxitron Image (bottom) of right tibia of Animal 5329 at four weeks. From the left, the proximal site, middle site and distal site contain Formulations Control, 10 and 9, respectively.

[0013] Figure 4. Gross Image (top) and Faxitron Image (bottom) of left tibia of Animal 5339 at eight weeks.

From the left, the proximal site, middle site and distal site contain Formulations 3, 2 and 1, respectively.

[0014] Figure 5. Gross Image (top) and Faxitron Image (bottom) of right tibia of Animal 5338 at eight weeks. From the left, the proximal site, middle site and distal site contain Formulations 6, 7 and 8, respectively.

[0015] Figure 6. Gross Image (top) and Faxitron Image (bottom) of right tibia of Animal 5340 at eight weeks. From the left, the proximal site, middle site and distal site contain Formulations Control, 10 and 9, respectively.

DETAILED DESCRIPTION OF THE INVENTION

[0016] In order that the invention herein described may be fully understood, the following detailed description is set forth.

[0017] "Amino acid sequence homology" is understood to include both amino acid sequence identity and similarity. Homologous sequences share identical and/or similar amino acid residues, where similar residues are conservative substitutions for, or "allowed point mutations" of, corresponding amino acid residues in an aligned reference sequence. Thus, a candidate polypeptide sequence that shares 70% amino acid homology with a reference sequence is one in which any 70% of the aligned residues are either identical to, or are conservative substitutions of, the corresponding residues in a reference sequence. Certain particularly preferred morphogenic polypeptides share at least 60%, and preferably 70% amino acid sequence identity with the C-terminal 102-106 amino acids, defining the conserved seven-cysteine domain of human OP-1, BMP-2, and related proteins.

[0018] Amino acid sequence homology can be determined by methods well known in the art. For instance, to

determine the percent homology of a candidate amino acid sequence to the sequence of the seven-cysteine domain, the two sequences are first aligned. The alignment can be made with, *e.g.*, the dynamic programming algorithm described in Needleman *et al.*, J. Mol. Biol., 48, pp. 443 (1970), and the Align Program, a commercial software package produced by DNAstar, Inc. The teachings of these references are incorporated herein by reference. An initial alignment can be refined by comparison to a multi-sequence alignment of a family of related proteins. Once the alignment is made and refined, a percent homology score is calculated. The aligned amino acid residues of the two sequences are compared sequentially for their similarity to each other. Similarity factors include similar size, shape and electrical charge. One particularly preferred method of determining amino acid similarities is the PAM250 matrix described in Dayhoff *et al.*, Atlas of Protein Sequence and Structure, 5, pp. 345-352 (1978 & Supp.), which is incorporated herein by reference. A similarity score is first calculated as the sum of the aligned pairwise amino acid similarity scores. Insertions and deletions are ignored for the purposes of percent homology and identity. Accordingly, gap penalties are not used in this calculation. The raw score is then normalized by dividing it by the geometric mean of the scores of the candidate sequence and the seven-cysteine domain. The geometric mean is the square root of the product of these scores. The normalized raw score is the percent homology.

[0019] "Bone" refers to a calcified (mineralized) connective tissue primarily comprising a composite of deposited calcium and phosphate in the form of hydroxyapatite, collagen (primarily Type I collagen) and bone cells such as osteoblasts, osteocytes and

osteoclasts, as well as bone marrow tissue which forms in the interior of true endochondral bone. Bone tissue differs significantly from other tissues, including cartilage tissue. Specifically, bone tissue is vascularized tissue composed of cells and a biphasic medium comprising a mineralized, inorganic component (primarily hydroxyapatite crystals) and an organic component (primarily of Type I collagen). Glycosaminoglycans constitute less than 2% of this organic component and less than 1% of the biphasic medium itself, or of bone tissue *per se*. Moreover, relative to cartilage tissue, the collagen present in bone tissue exists in a highly-organized parallel arrangement. Bony defects, whether from degenerative, traumatic or cancerous etiologies, pose a formidable challenge to the reconstructive surgeon. Particularly difficult is reconstruction or repair of skeletal parts that comprise part of a multi-tissue complex, such as occurs in mammalian joints.

[0020] "Bone formation" refers to formation of endochondral bone or formation of intramembranous bone. In humans, bone formation begins during the first 6-8 weeks of fetal development. Progenitor stem cells of mesenchymal origin migrate to predetermined sites, where they either: (a) condense, proliferate, and differentiate into bone-forming cells (osteoblasts), a process observed in the skull and referred to as "intramembranous bone formation" or, (b) condense, proliferate and differentiate into cartilage-forming cells (chondroblasts) as intermediates, which are subsequently replaced with bone-forming cells. More specifically, mesenchymal stem cells differentiate into chondrocytes. The chondrocytes then become calcified, undergo hypertrophy and are replaced by newly formed bone

made by differentiated osteoblasts, which now are present at the site. Subsequently, the mineralized bone is extensively remodeled, thereafter becoming occupied by an ossicle filled with functional bone-marrow elements. This process is observed in long bones and referred to as "endochondral bone formation". In postfetal life, bone has the capacity to repair itself upon injury by mimicking the cellular process of embryonic endochondral bone development. That is, mesenchymal progenitor stem cells from the bone marrow, periosteum, and muscle can be induced to migrate to the defect site and begin the cascade of events described above. There, they accumulate, proliferate, and differentiate into cartilage, which is subsequently replaced with newly formed bone.

[0021] "Bone morphogenic protein (BMP)" refers to a protein belonging to the BMP family of the TGF-β superfamily of proteins (BMP family) based on DNA and amino acid sequence homology. A protein belongs to the BMP family according to this invention when it has at least 50% amino acid sequence identity with at least one known BMP family member within the conserved C-terminal cysteine-rich domain which characterizes the BMP protein family. Members of the BMP family may have less than 50% DNA or amino acid sequence identity overall.

[0022] "Bone precursor composition" refers to a composition comprising a cement mixture and a pore-forming agent. Preferably, the bone precursor composition is biocompatible and bioabsorbable. The composition can be used to form a cement matrix for bone implant to form, repair or replace damaged connective tissue such as bone tissue.

[0023] "Binder" refers to any biocompatible material which, when admixed with the bone precursor composition

and/or osteogenic protein, provides desired handling properties without adversely affecting bone formation. As taught herein, the skilled artisan can determine an effective amount of protein for use with any suitable binder using only routine experimentation. Among the other characteristics of a preferred binder is an ability to render the device: pliable, shapeable and/or malleable; injectable; adherent to bone, cartilage, muscle and other tissues, resistant to disintegration upon washing and/or irrigating during surgery; and, resistant to dislodging during surgery, suturing and post-operatively, to name but a few.

[0024] "Biocompatible" refers to a material that does not elicit detrimental effects associated with the body's various protective systems, such as cell and humoral-associated immune responses, e.g., inflammatory responses and foreign body fibrotic responses. The term biocompatible also implies that no specific undesirable cytotoxic or systemic effects are caused by the material when it is implanted into the patient.

[0025] "Cement mixture" refers to a mixture that is the precursor of solid cement. The mixture can be in a dry powder or granular form. Upon mixing with a liquid initiator, the mixture forms a plastic paste. The paste undergoes a chemical reaction and/or a crystal rearrangement and hardens with time into a cured solid cement as a result of the hydration reaction. The liquid initiator can be a physiologically acceptable aqueous initiator, e.g., water, an aqueous buffer or an aqueous solution. The cement mixture can be used as a joiner, or filler for the assembly of connective tissue surfaces (e.g., bone tissue), which are not in direct contact, and to bond bone tissue to metallic or synthetic prosthetic devices. Preferably, the cement mixture is a calcium

cement mixture. More preferably, the mixture is a calcium phosphate cement mixture, calcium sulfate cement mixture such as calcium sulfate hemihydrate, or a combination thereof.

[0026] "Calcium phosphate cement mixture" refers to a cement precursor composition that comprises at least two calcium phosphate compounds selected from the group consisting of calcium phosphate, amorphous calcium phosphate, decarbonated amorphous calcium phosphate, beta-tricalcium phosphate, alpha-tricalcium phosphate, monocalcium phosphate, dicalcium phosphate, octacalcium phosphate, calcium metaphosphate, heptacalcium phosphate, calcium pyrophosphate. The calcium phosphate cement mixture forms calcium phosphate cement upon hydration and hardening.

[0027] "Calcium sulfate cement mixture" refers to a cement precursor composition that comprises a form of calcium sulfate including but not limited to calcium sulfate, calcium sulfate hemihydrate (Plaster of Paris) and calcium sulfate dihydrate (gypsum). The calcium sulfate cement mixture forms calcium sulfate cement upon hydration and hardening.

[0028] "Cement matrix" refers to a composition that forms after mixing the bone precursor composition of the invention with the liquid initiator. The cement matrix may be in a moldable putty form ready for implant or in a hardened solid form already implanted *in vivo* or *in situ*. The hardened solid form has a scaffolding structure on which infiltrating cells can attach, proliferate and participate in the morphogenic process culminating in bone formation. The cement matrix may also contain one or more components selected from a binder and a bioactive agent such as BMP.

[0029] "Conservative substitutions" refers to residues that are physically or functionally similar to the corresponding reference residues. That is, a conservative substitution and its reference residue have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff *et al., supra*. Examples of conservative substitutions are substitutions within the following groups: (a) valine, glycine; (b) glycine, alanine; (c) valine, isoleucine, leucine; (d) aspartic acid, glutamic acid; (e) asparagine, glutamine; (f) serine, threonine; (g) lysine, arginine, methionine; and (h) phenylalanine, tyrosine. The term "conservative variant" or "conservative variation" also includes the use of a substituting amino acid residue in place of an amino acid residue in a given parent amino acid sequence, where antibodies specific for the parent sequence are also specific for, i.e., "cross-react" or "immuno-react" with, the resulting substituted polypeptide sequence.

[0030] "Defect" or "defect site" refers to a site requiring bone, joint, cartilage or ligament repair, construction, fusion, regeneration or augmentation. The site may be an orthopedic structural disruption or abnormality, or a site where bone does not normally grow. The defect further can define an osteochondral defect, including a structural disruption of both the bone and overlying cartilage. A defect can assume the configuration of a "void", which is understood to mean a three-dimensional defect such as, for example, a gap, cavity, hole or other substantial disruption in the structural integrity of a bone or joint. A defect can be the result of accident, disease, surgical manipulation,

and/or prosthetic failure. In certain embodiments, the defect is a void having a volume incapable of endogenous or spontaneous repair. Such defects in long bone are generally twice the diameter of the subject bone and are also called "critical size" defects. For example, in a canine ulna defect model, the art recognizes such defects to be approximately 3-4 cm. Generally, critical size defects are approximately 1.0 cm, and incapable of spontaneous repair. See, for example, Schmitz *et al.*, Clinical Orthopaedics and Related Research, 205, pp. 299-308 (1986); and Vukicevic *et al.*, in Advances in Molecular and Cell Biology, 6, pp. 207-224 (1993)(JAI Press, Inc.). In rabbit and monkey segmental defect models, the gap is approximately 1.5 cm and 2.0 cm, respectively. In other embodiments, the defect is a non-critical size segmental defect. Generally, these are capable of spontaneous repair. In certain other embodiments, the defect is an osteochondral defect, such as an osteochondral plug. Such a defect traverses the entirety of the overlying cartilage and enters, at least in part, the underlying bony structure. In contrast, a chondral or subchondral defect traverses the overlying cartilage, in part or in whole, respectively, but does not involve the underlying bone. Other defects susceptible to repair using the instant invention include, but are not limited to, non-union fractures; bone cavities; tumor resection; fresh fractures (distracted or undistracted); cranial, maxillofacial and facial abnormalities, for example, in facial skeletal reconstruction, specifically, orbital floor reconstruction, augmentation of the alveolar ridge or sinus, periodontal defects and tooth extraction socket; cranioplasty, genioplasty, chin augmentation, palate reconstruction, and other large bony reconstructions;

vertebroplasty, interbody fusions in the cervical, thoracic and lumbar spine and posteriolateral fusions in the thoracic and lumbar spine; in osteomyelitis for bone regeneration; appendicular fusion, ankle fusion, total hip, knee and joint fusions or arthroplasty; correcting tendon and/or ligamentous tissue defects such as, for example, the anterior, posterior, lateral and medial ligaments of the knee, the patella and achilles tendons, and the like as well as those defects resulting from diseases such as cancer, arthritis, including osteoarthritis, and other bone degenerative disorders such as osteochondritis dessicans.

[0031] "Morphogenic protein" refers to a protein having morphogenic activity. Preferably a morphogenic protein of this invention comprises at least one polypeptide belonging to the BMP protein family. Morphogenic proteins may be capable of inducing progenitor cells to proliferate and/or to initiate differentiation pathways that lead to cartilage, bone, tendon, ligament, neural or other types of tissue formation depending on local environmental cues, and thus morphogenic proteins may behave differently in different surroundings. For example, an osteogenic protein may induce bone tissue at one treatment site and neural tissue at a different treatment site.

[0032] "Osteogenic protein (OP)" refers to a morphogenic protein that is capable of inducing a progenitor cell to form cartilage and/or bone. The bone may be intramembranous bone or endochondral bone. Most osteogenic proteins are members of the BMP protein family and are thus also BMPs. As described elsewhere herein, the class of proteins is typified by human osteogenic protein (hOP-1). Other osteogenic proteins useful in the practice of the invention include osteogenically active

forms of OP-1, OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, TGF-β and conservative amino acid sequence variants thereof having osteogenic activity. In one currently preferred embodiment, osteogenic protein includes any one of: OP-1, OP-2, OP-3, BMP-2, BMP-4, BMP-5, BMP-6, BMP-9, and amino acid sequence variants and homologs thereof, including species homologs thereof. Particularly preferred osteogenic proteins are those comprising an amino acid sequence having at least 70% homology with the C-terminal 102-106 amino acids, defining the conserved seven cysteine domain, of human OP-1, BMP-2, and related proteins. Certain preferred embodiments of the instant invention comprise the osteogenic protein, OP-1. As further described elsewhere herein, the osteogenic proteins suitable for use with applicants' invention can be identified by means of routine experimentation using the art-recognized bioassay described by Reddi and Sampath (Sampath *et al.*, Proc. Natl. Acad. Sci., 84, pp. 7109-13, incorporated herein by reference)

[0033] Proteins useful in this invention include eukaryotic proteins identified as osteogenic proteins (see U.S. Patent 5,011,691, incorporated herein by reference), such as the OP-1, OP-2, OP-3 and CBMP-2 proteins, as well as amino acid sequence-related proteins, such as DPP (from Drosophila), Vg1 (from Xenopus), Vgr-1 (from mouse), GDF-1 (from humans, see Lee, PNAS, 88, pp. 4250-4254 (1991)), 60A (from Drosophila, see Wharton *et al.* PNAS, 88, pp. 9214-9218

(1991)), dorsalin-1 (from chick, see Basler *et al*. Cell 73, pp. 687-702 (1993) and GenBank accession number L12032) and GDF-5 (from mouse, see Storm *et al*. Nature, 368, pp. 639-643 (1994)). The teachings of the above references are incorporated herein by reference. BMP-3 is also preferred. Additional useful proteins include biosynthetic morphogenic constructs disclosed in U.S. Pat. No. 5,011,691, incorporated herein by reference, e.g., COP-1, COP-3, COP-4, COP-5, COP-7 and COP-16, as well as other proteins known in the art. Still other proteins include osteogenically active forms of BMP-3b (see Takao, *et al*. Biochem. Biophys. Res. Comm., 219, pp. 656-662 (1996)). BMP-9 (see WO95/33830), BMP-15 (see WO96/35710), BMP-12 (see WO95/16035), CDMP-1 (see WO 94/12814), CDMP-2 (see WO94/12814), BMP-10 (see WO94/26893), GDF-1 (see WO92/00382), GDF-10 (see WO95/10539), GDF-3 (see WO94/15965) and GDF-7 (see WO95/01802). The teachings of the above references are incorporated herein by reference.

[0034] "Repair" refers to new bone and/or cartilage formation which is sufficient to at least partially fill the void or structural discontinuity at the defect. Repair does not, however, mean, or otherwise necessitate, a process of complete healing or a treatment which is 100% effective at restoring a defect to its pre-defect physiological/structural/mechanical state.

[0035] "Solid cement" refers to the cured, hardened solid as a result of the hydration reaction of the cement mixture. Depending on the composition of the cement mixture, it can take from a few minutes to a few hours for the cement mixture to form a solid cement. The solid cement is preferably calcium phosphate cement or calcium sulfate cement. The calcium phosphate cement can be in a form of hydroxyapatite such as hydroxyapatite, carbonated

hydroxyapatite or poorly-crystalline hydroxyapatite. The solid cement can be formed *in vivo* or *in situ* to induce bone growth, repair or replace damaged connective tissue such as bone tissue.

Bone Precursor Composition

[0036] The present invention provides a bone precursor composition comprising a cement mixture and a pore-forming agent. Preferred cement mixtures are calcium cement mixtures. More preferably, the calcium cement mixture is a calcium phosphate cement mixture or a calcium sulfate cement mixture. Upon hydration and setting at ambient temperature, e.g., room or body temperature, the cement mixture forms a solid cement. Hydration can be achieved by the addition of liquid such as water, saline buffer or an aqueous solution. The setting time for these cements may range from a few minutes to a few hours depending on their composition and the amount of liquid added. Buffers such as sodium phosphate or sodium pyrophosphate can reduce the setting time. The *in situ* hardening ensures a proper cohesion of the implant until its complete replacement by new bone, and the rapid adsorption of the pore-forming agents allow cellular factors to penetrate the cement matrix.

[0037] In one embodiment, the calcium phosphate cement mixture is selected from the group consisting of a mixture of beta-tricalcium phosphate (β-TCP) and monocalcium phosphate monohydrate (MCPM); a mixture of β-TCP, dicalcium phosphate dihydrate (DCPD) and calcium carbonate (CC); a mixture of monocalcium phosphate, tricalcium phosphate and calcium carbonate; a mixture of a decarbonated amorphous calcium phosphate and a second calcium phosphate; a mixture of tetracalcium phosphate (TTCP) and a second calcium phosphate.

[0038] In one embodiment the second calcium phosphate is selected from the group consisting of monocalcium phosphate, dicalcium phosphate anhydrous, dicalcium phosphate dihydrate, calcium metaphosphate, heptacalcium phosphate, calcium pyrophosphate, alpha-tricalcium phosphate, beta-tricalcium phosphate, octacalcium phosphate and amorphous calcium phosphate.

[0039] In a preferred embodiment, the calcium phosphate cement mixture comprises a mixture of beta-tricalcium phosphate (β-TCP) and monocalcium phosphate monohydrate (MCPM). More preferably, the β-TCP/MCPM mixture further comprises calcium pyrophosphate (CPP), calcium sulfate dihydrate (CSD) and calcium sulfate hemihydrate (CSH). Upon mixing the β-TCP with MCPM, small crystals of DCPD are formed throughout the paste. These crystals act as bridges between the β-TCP particles. The addition of CPP, CSD and CSH increases the setting time from 30 seconds to about 10 minutes.

[0040] In another preferred embodiment, the calcium phosphate cement mixture comprises a mixture of β-TCP, dicalcium phosphate dihydrate (DCPD) and calcium carbonate (CC). Preferably, the β-TCP/DCPD/CC mixture further comprises hydroxyapatite. In the presence of water, the mixture produces hydroxyapatite. The setting time of the β-TCP, DCPD and CC mixture can be reduced from 4.5 hours to about 20 minutes by the addition of small amounts (8% (w/w)) of hydroxyapatite. In yet another preferred embodiment, the calcium phosphate cement mixture comprises a mixture of monocalcium phosphate, tricalcium phosphate and calcium carbonate. Under physiologic conditions, carbonated hydroxyapatite is formed within minutes from this cement mixture in a nonexothermic reaction.

[0041] In a most preferred embodiment, the calcium phosphate cement mixture comprises a mixture of decarbonated amorphous calcium phosphate and a second calcium phosphate, wherein the second calcium phosphate is selected from the group consisting of dicalcium phosphate dihydrate, calcium metaphosphate, heptacalcium phosphate, calcium pyrophosphate and tricalcium phosphate. The decarbonated amorphous calcium phosphate is formed by heating an amorphous carbonated calcium phosphate to remove a portion of the carbonate component. The amorphous carbonated calcium phosphate is precipitated from an aqueous solution comprising calcium ions, phosphate ions and carbonate ions having a calcium to phosphorous ratio in the range of about 1.55 to 1.7. At body temperature (37 °C), mixing the decarbonated amorphous calcium phosphate and a second calcium phosphate in water or buffer results in a poorly-crystalline hydroxyapatite which forms within 20 minutes. Poorly-crystalline hydroxyapatite contains nanometer-sized crystals and has substantially the same X-ray diffraction spectrum as bone. Compared to other crystalline cements, the poorly-crystalline cement is more soluble and provides better osteoconductive cell mediated absorption. The solubility of the poorly-crystalline hydroxyapatite can be further improved by modifying the Ca/P ratio. The poorly-crystalline cement is particularly suitable for the incorporation of bioactive agents, such as BMPs since the setting reaction produces minimal heat, which minimizes the denaturing of protein structure.

[0042] In a most preferred embodiment, the calcium phosphate cement mixture is tetracalcium phosphate (TTCP) and a second calcium phosphate; wherein the second calcium phosphate is selected from the group consisting

of monocalcium phosphate, dicalcium phosphate anhydrous (DCPA), dicalcium phosphate dihydrate (DCPD), alpha-tricalcium phosphate, beta-tricalcium phosphate, octacalcium phosphate, and amorphous calcium phosphate. When the compounds are mixed together with water, an isothermic reaction occurs, resulting in the cement setting within 15 to 30 minutes. The cement then fully converts to a solid mass of hydroxyapatite *in situ* within 4-6 hours. It is preferred that the tetracalcium phosphate has a molar Ca/P ratio below 2:1. If the ratio is above 2:1, calcium oxide may be present as an impurity. This causes the pH of the cement slurry to rise substantially above pH 8.5, which impedes the setting reaction. It is critical to maintain the tetracalcium phosphate under anhydrous conditions. If not, the compound will be less reactive in forming cement. In a preferred embodiment, the second calcium phosphate is selected from the group consisting of dicalcium phosphate anhydrous (DCPA) and dicalcium phosphate dihydrate (DCPD). Relatively large TTCP and small DCPA particles help to achieve rapid setting and high strength in the cement. In forming the cement, the TTCP/DCPA ratio can range from 1:1 to 1:4. Preferably, the ratio is 1:1. A 1:1 ratio produces hydroxyapatite.

[0043] In another most preferred embodiment, the calcium sulfate cement mixture used in the invention comprises calcium sulfate hemihydrate (CSH, $CaSO_4.1/2H_20$). CSH is produced by heating gypsum ($CaSO_4.2H_20$) so that it loses 75% of its water. When CSH is mixed with water, a paste is formed that rapidly solidifies. CSH has been shown to be biocompatible, as it does not incite local inflammatory or foreign body responses. In addition, osteoblasts are able to attach to the CSH, and osteoclasts can actively resorb it. It is preferred that

a medical grade CSH that has uniform size and shape is used for the bone precursor composition. The medical grade CSH exhibits a slower, more predictable solubility and resorption.

[0044] The invention also provides a composition comprising a solid cement formed from the cement mixture and a pore-forming agent. Preferably, the solid cement is a calcium phosphate cement or a calcium sulfate cement. Calcium phosphate cements include but are not limited to hydroxyapatite, poorly-crystalline HA cement or carbonated hydroxyapatite.

[0045] The pore-forming agents of this invention may be in bead or resin form. The pore-forming agents can be resorbable biocompatible polymers including both natural and synthetic polymers. Natural polymers are typically absorbed by enzymatic degradation in the body, while synthetic resorbable polymers typically degrade by a hydrolytic mechanism.

[0046] In one embodiment, the pore-forming agent is selected from the group consisting of ethylenevinylacetate, natural and synthetic collagen, poly(glaxanone), poly(phosphazenes), polyglactin, polyglactic acid, polyaldonic acid, polyacrylic acids, polyalkanoates, polyorthoesters, poly(L-lactide) (PLLA), poly(D,L-lactide) (PDLLA), polyglycolide (PGA), poly(lactide-co-glycolide (PLGA), poly(ζ-caprolactone), poly(trimethylene carbonate), poly(p-dioxanone), poly(ζ-caprolactone-co-glycolide), poly(glycolide-co-trimethylene carbonate) poly(D,L-lactide-co-trimethylene carbonate), polyarylates, polyhydroxybutyrate (PHB), polyanhydrides, poly(anhydride-co-imide) and co-polymers thereof, polymers of amino acids, propylene-co-fumarates, a polymer of one or more α-hydroxy carboxylic acid

monomers, calcium sulfate, bioactive glass compositions, admixtures thereof and any derivatives and modifications thereof; with the proviso that when the cement mixture is calcium sulfate hemihydrate, the pore-forming agent is not calcium sulfate. Preferably, the modification of the pore-forming agent is less than 50% of the overall structure.

[0047] In a more preferred embodiment, the pore-forming agent is selected from the group consisting of polyorthoesters, poly(L-lactide) (PLLA), poly(D,L-lactide) (PDLLA), polyglycolide (PGA), poly(lactide-co-glycolide (PLGA), poly(ζ-caprolactone), poly(trimethylene carbonate), poly(p-dioxanone), poly(ζ-caprolactone-co-glycolide), poly(glycolide-co-trimethylene carbonate), poly(D,L-lactide-co-trimethylene carbonate), polyarylates and co-polymers thereof.

[0048] In another more preferred embodiment, the pore-forming agent is selected from the group consisting of ethylenevinylacetate, natural and synthetic collagen, poly(glaxanone), poly(phosphazenes), polyglactin, polyglactic acid, polyaldonic acid, polyacrylic acids, polyalkanoates and co-polymers thereof.

[0049] In yet another more preferred embodiment the pore-forming agent is selected from the group consisting of polyhydroxybutyrate (PHB), anhydrides including polyanhydrides, poly(anhydride-co-imide) and co-polymers thereof, polymers of amino acids, propylene-co-fumarates, a polymer of one or more -hydroxy carboxylic acid monomers, (e.g., α-hydroxy acetic acid (glycolic acid) and/or α-hydroxy propionic acid (lactic acid)), calcium sulfate and bioactive glass compositions. α-hydroxy propionic acid can be employed in its d- or l- form, or as a racemic mixture.

[0050] In an even more preferred embodiment the pore-forming agent is selected from the group consisting of poly(lactide-co-glycolide) (PLGA) and calcium sulfate. Depending upon the desired rate to form pores in the calcium cement, the molar ratio of the lactide, glycolide monomers can be adjusted. In a preferred embodiment, the monomer ratio is 50:50. In general, the higher the molecular weight, the slower the biodegradation. Preferably, the molecular weight range of the polymer is from about 5,000 to 100,000 daltons, more preferably 10,000 to 30,000 daltons. PLGA has a more rapid resorption rate than calcium sulfate. The degradation of PLGA is through chemical hydrolysis of the hydrolytically unstable backbone.

[0051] In a most preferred embodiment, the calcium phosphate cement mixture comprises a mixture of tetracalcium phosphate and dicalcium phosphate anhydrous; and the pore-forming agent is selected from the group consisting of PLGA and calcium sulfate. In another most preferred embodiment, the calcium sulfate cement mixture comprises calcium sulfate hemihydrate; and the pore-forming agent is PLGA.

[0052] The resorption rate of the pore-forming agent is faster than that of the nonresorbable calcium cement. As a result, pores are formed in the calcium cement. The rate of resorption of the pore-forming agent is dependent on the proportion, polymer type and particle size of the pore-forming agent. The mechanical strength of the implant decreases as the proportion of the pore-forming agent increases. An excessive amount of pore-forming agent leads to a decrease in the density of the cement body which results in lower mechanical strength. A deficiency in the amount of pore-forming agent may result in insufficient pores in the cement. In a preferred

embodiment, the proportion of pore-forming agent is 10-70% by volume, more preferably, 40-60% by volume. The proportion of pore-forming agent is preferably 7-40% by weight, more preferably 7-25% by weight, most preferably 7-14% by weight when the pore-forming agent is PLGA.

[0053] The particle size of the pore-forming agent will influence the pore size generated in the cement. A sufficient pore size is required to provide residence spaces for the infiltrating osteolytic cells and osteoblasts. The pore size also controls the rate of resorption. Bone repair is optimal when the rate of resorption coincides with the rate of bone growth. It is preferred that the pore-forming agent creates a pore size diameter of 20-500 μm, preferably 20-140 μm, more preferably 50-140 μm, and most preferably 75-140 μm. When the pore-forming agent is PLGA, the particle size is preferably 20-140 μm or 310-500 μm. When the pore-forming agent is calcium sulfate, the particle size is preferably 20-140 μm or 260-500 μm.

[0054] The bone precursor composition of this invention may also be combined with one or more bioactive agents. The bioactive agent may be an agent that enhances bone growth. In one embodiment, the bioactive agent is a bone morphogenic protein. In a more preferred embodiment, the bone morphogenic protein is selected from the group consisting of OP-1 (BMP-7), OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, and TGF-β. In a most preferred embodiment, the morphogenic protein is OP-1.

[0055] In a most preferred embodiment, the bioactive agent is encapsulated in the pore-forming agent. As the pore-forming agent is slowly resorbed by the osteoclast cells, the encapsulated bioactive agent is gradually released into the matrix. At the implant site, one may deliver the bioactive agent through a combination of different biodegradable agents, preferably, differing in the rate of resorption, to achieve a multiple boost delivery system. In another preferred embodiment, the biodegradable agent is multi-layered. Each layer comprises a different biodegradable agent, preferably, differing in the rate of resorption. Methods of encapsulating the bioactive agent include but are not limited to the emulsion-solvent evaporation method (Grandfils *et al.*, Journal of Biomedical Materials Research, 26, pp. 467-479 (1992)) and the method described in Herbert *et al.*, Pharmaceutical Research, 15, pp. 357-361 (1998). The above two references are incorporated herein by reference. The latter method is especially suitable for encapsulating proteins. Other methods are described in U.S. patents 6,110,503, 5,654,008 and 5,271,961, which are incorporated herein by reference. In a preferred embodiment, the bioactive agent is stabilized by the addition of lactose during the encapsulation process.

[0056] In another preferred embodiment, the bioactive agent is a repair cell. In a preferred embodiment, the repair cell is a mammalian cell, more preferably, a human cell of the same type as that of the tissue being repaired or reconstructed. Suitable examples of repair cells include bone cells such as bone marrow stem cells, osteocytes, osteoblasts, osteoclasts and bone progenitor cells. In another embodiment, the cell is transfected with a nucleic acid molecule encoding a BMP.

[0057] In yet another preferred embodiment, the bioactive agent is a nucleic acid molecule comprising a sequence encoding a BMP, preferably, OP-1 (SEQ ID NO: 10). In a preferred embodiment, the nucleic acid molecule is a RNA or DNA molecule. The nucleic acid sequence encoding the BMP may be inserted in recombinant expression vectors. Examples of vectors include but are not limited to pBR322, pH717, pH731, pH752, pH754 and pW24. SP6 vectors may be used for *in vitro* transcription of RNA. Transcription promoters useful for expressing the BMP include but are not limited to the SV40 early promoter, the adenovirus promoter (AdMLP), the mouse metallothionein-I promoter (mMT-I), the Rous sarcoma virus (RSV) long terminal repeat (LTR), the mouse mammary tumor virus long terminal repeat (MMTV-LTR), and the human cytomegalovirus major intermediate-early promoter (hCMV). The DNA sequences for all of these promoters are known in the art and are available commercially. The DNA sequence may also be inserted in the genome of a recombinant virus such as, for example recombinant adenovirus, adeno-associated virus or retrovirus. The repair cell or bone progenitor cell is then transfected or infected with the vector or virus to express the BMP protein. The nucleic acid sequence may transiently or stably transfect the repair cell or bone progenitor cell.

[0058] In one embodiment, the nucleic acid molecule is directly injected into the implant site. In another embodiment, the nucleic acid molecule is encapsulated in the pore-forming agent, preferably, in PLGA of 25-30 kD. Preferably, the nucleic acid molecule is trapped in a carrier selected from the group consisting of mannitol, sucrose, lactose, trehalose, liposomes, proteoliposomes that contain viral envelope proteins and polylysine-glycoprotein complexes. See, *e.g.*, Ledley, J. Pediatrics

110, pp. 1 (1987); Nicolau *et al.*, Proc. Natl. Acad. Sci. U.S.A., 80, pp. 1068 (1983). In another preferred embodiment, the nucleic acid is transfected or infected into target cells such as bone progenitor cells and repair cells that have been removed from the body. The transfected cells are then re-implanted into the body.

Method of producing the bone precursor composition and solid cement

[0059] The invention also provides a method of producing the bone precursor composition. The cement mixture in dried powdered form is blended with the pore-forming agent to form an evenly distributed mixture of the bone precursor composition. This allows the cement mixture to surround the pore-forming agents so that pores are formed in the cement matrix *in situ*. Water, buffer or an aqueous solution is then added to the composition. The paste is allowed to set and harden to form a composition comprising a pore-forming agent dispersed in a solid cement. This method is distinguishable from methods of preparing formulations wherein the cement mixture is added to a biodegradable material in liquid form. There, the biodegradable material serves as a matrix around the cement instead of a pore-forming agent.

Binder

[0060] The bone precursor composition of this invention may further be combined with a biocompatible binder. The binder provides enhanced viscosity and cohesiveness of the composition, allowing the skilled practitioner to position and shape the composition within the voids, defects or other areas in which new bone growth is desired. The enhanced cohesiveness of the composition also prevents particle migration associated

with grafting materials for orthopedic, maxillofacial and dental applications. The minimum amount of binder is that amount required to give easy formability and provide sufficient particle cohesion and shape retention during the period of tissue ingrowth.

[0061] The binder according to this invention is biodegradable, biocompatible and has fluid flow properties. The binders contemplated as useful herein include, but are not limited to art-recognized suspending agents, viscosity-producing agents, gel-forming agents and emulsifying agents. Other binders include agents used to suspend ingredients for topical, oral or parental administration. Yet other binders are agents useful as tablet binders, disintegrants or emulsion stabilizers. Still other binders are agents used in cosmetics, toiletries and food products (See USP XXII -NF XVII The Nineteen Ninety U.S. Pharmacopeia and the National Formulary (1990)). Other components including antioxidants such as EDTA, citrate, butylated hydroxytoluene (BHT), and surfactants such as poly(sorbates) and poly(oxyethylenes) may be added to the binder.

[0062] A preferred binder is carboxymethylcellulose (CMC) sodium. Carboxymethylcellulose sodium is the sodium salt of a polycarboxymethyl ether of cellulose with a typical molecular weight ranging from 90,000 - 700,000 daltons.

[0063] Aside from binders that are flowable at room temperature, binders also include reagents such as gelatin, that are solubilized in warm or hot aqueous solutions, and are transformed into a non-flowable gel upon cooling. The gelatin composition is formulated so that the composition is flowable at temperatures above body temperature of the mammal for implant, but

transitions to relatively non-flowable gel at or slightly above such body temperature.

[0064] It is preferred that the binder is selected from the group consisting of sodium alginate, hyaluronic acid, sodium hyaluronate, gelatin, peptides, mucin, chrondroitin sulfate, chitosan, poloxamer, glycosaminoglycan, polysaccharide, polyethylene glycol, methylcellulose, carboxy methylcellulose, carboxy methylcellulose sodium, carboxy methylcellulose calcium, hydroxypropyl methylcellulose, hydroxybutyl methylcellulose, hydroxyethyl methylcellulose, hydroxyethylcellulose, methylhydroxyethyl cellulose, hydroxyethyl cellulose, mannitol, white petrolatum, mannitol/dextran combinations, mannitol/white petrolatum combinations, sesame oil, fibrin glue, blood and admixtures thereof.

[0065] More preferably, the binder is selected from the group consisting of sodium alginate, hyaluronic acid, methylcellulose, carboxy methylcellulose, carboxy methylcellulose sodium, carboxy methylcellulose calcium, hydroxypropyl methylcellulose, hydroxybutyl methylcellulose, hydroxyethyl methylcellulose, hydroxyethylcellulose, methylhydroxyethyl cellulose, hydroxyethyl cellulose and admixtures thereof. Most preferably, the binder is selected from the group consisting of sodium alginate, hyaluronic acid, carboxy methylcellulose, carboxy methylcellulose sodium and carboxy methylcellulose calcium.

[0066] In one embodiment, the binder of this invention is selected from a class of high molecular weight hydrogels including sodium hyaluronate (~ 500-3000 kD), chitosan (~ 100 - 300 kD), poloxamer (~ 7 - 18 kD), and glycosaminoglycan (~ 2000 - 3000 kD). In a preferred embodiment, the glycosaminoglycan is

N,O-carboxymethylchitosan glucosamine. Hydrogels are cross-linked hydrophilic polymers in the form of a gel which have a three-dimensional network. Hydrogel may carry a net positive charge, a net negative charge, or may be neutral. A typical net negative charged hydrogel is alginate. Hydrogels carrying a net positive charge may be typified by extracellular matrix components such as collagen and laminin. Examples of commercially available extracellular matrix components include Matrigel™ (Dulbecco's modified eagle's medium with 50 µg/ml gentamicin) and Vitrogen™ (a sterile solution of purified, pepsin-solubilized bovine dermal collagen dissolved in 0.012 N HCl). Examples of a net neutral hydrogel is highly crosslinked polyethylene oxide and polyvinyalcohol.

[0067] In another preferred embodiment, the binder is polyethylene glycol. A mixture of low- and high-molecular-weight polyethylene glycols produce a paste with the proper viscosity. For example, a mixture of polyethylene glycols of molecular weight 400-600 daltons and 1500 daltons at the proper ratio are effective in forming a binder according to this invention.

[0068] In yet another embodiment, the binder is selected from a class of polysaccharides with an average molecular weight of about 200,000 to 5,000,000 daltons consisting of dextran, dextran sulfate, diethylaminoethyl dextran, dextran phosphate or mixtures thereof. Lower molecular weight polysaccharides have a faster dextran absorption rate, which results in earlier exposure of the porous bone precursor composition material. If it is desired that dextrans remain in the site for an extended period, dextrans of relatively high molecular weight may be used. Other preferred polysaccharides include starch, fractionated starch, amylopectin, agar, gum arabic,

pullullan, agarose, carrageenan, dextrins, fructans, inulin, mannans, xylans, arabinans, glycogens, glucans, xanthan gum, guar gum, locust bean gum, tragacanth gum, karaya gum, and derivatives and mixtures thereof.

[0069] In another preferred embodiment, the binder is selected from the group consisting of mannitol, white petrolatum, mannitol/dextran combinations, mannitol/white petrolatum combinations, sesame oil, fibrin glue and admixtures thereof. In a more preferred embodiment, the binder is fibrin glue. Fibrin glue comprises a mixture of mammalian fibrinogen and thrombin. Human fibrinogen is commercially available in products such as, but not limited to Tissucol® (Immuno AG, Vienna, Austria), Beriplast® (Behringwerke, Marburg, Germany), Biocoll® (Centre de Transfusion Sanguine de Lille, Pours, France) and Transglutine® (CNTS Fractionation Centre, Strasbourg, France). Fibrin glue may also be made of fibrinogen and thrombin from other mammalian sources, such as, for example, bovine and murine sources.

[0070] In yet another preferred embodiment, the binder is human blood, preferably autogenous blood. This kind of binder serves as a protein-sequestering material. When added to the bone precursor composition of this invention that contains a BMP, the blood clots to form a malleable composite. This allows the BMP to be sequestered within the cement matrix for a period of time sufficient to allow the protein to increase the otherwise natural rate of osteogenic activity of the infiltrating mammalian progenitor cells.

[0071] The invention also relates to a kit for bone implant comprising the bone precursor composition material of the invention and a bioactive agent such as bone morphogenic protein. In one embodiment, the kit further comprises a binder. In another embodiment, the

kit comprises the bone precursor composition material of the invention and a binder.

Bone Morphogenic Protein Family

[0072] The BMP family, named for its representative bone morphogenic/osteogenic protein family members, belongs to the TGF-β protein superfamily. Of the reported "BMPs" (BMP-1 to BMP-18), isolated primarily based on sequence homology, all but BMP-1 remain classified as members of the BMP family of morphogenic proteins (Ozkaynak *et al.*, EMBO J., 9, pp. 2085-93 (1990)).

[0073] The BMP family includes other structurally-related members which are morphogenic proteins, including the *drosophila* decapentaplegic gene complex (DPP) products, the Vg1 product of *Xenopus laevis* and its murine homolog, Vgr-1 (see, *e.g.*, Massagué, Annu. Rev. Cell Biol., 6, pp. 597-641 (1990), incorporated herein by reference).

[0074] The C-terminal domains of BMP-3, BMP-5, BMP-6, and OP-1 (BMP-7) are about 60% identical to that of BMP-2, and the C-terminal domains of BMP-6 and OP-1 are 87% identical. BMP-6 is likely the human homolog of the murine Vgr-1 (Lyons *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86, pp. 4554-59 (1989)); the two proteins are 92% identical overall at the amino acid sequence level (U. S. Patent No. 5,459,047, incorporated herein by reference). BMP-6 is 58% identical to the *Xenopus* Vg-1 product.

Biochemical Structural and Functional Properties of Bone Morphogenic Proteins

[0075] The naturally occurring bone morphogens share substantial amino acid sequence homology in their C-terminal regions (domains). Typically, the above-

mentioned naturally occurring osteogenic proteins are translated as a precursor, having an N-terminal signal peptide sequence typically less than about 30 residues, followed by a "pro" domain that is cleaved to yield the mature C-terminal domain of approximately 100-140 amino acids. The signal peptide is cleaved rapidly upon translation, at a cleavage site that can be predicted in a given sequence using the method of Von Heijne Nucleic Acids Research, 14, pp. 4683-4691 (1986). The pro domain typically is about three times larger than the fully processed mature C-terminal domain.

[0076] Another characteristic of the BMP protein family members is their apparent ability to dimerize. Several bone-derived osteogenic proteins (OPs) and BMPs are found as homo- and heterodimers in their active forms. The ability of OPs and BMPs to form heterodimers may confer additional or altered morphogenic inductive capabilities on morphogenic proteins. Heterodimers may exhibit qualitatively or quantitatively different binding affinities than homodimers for OP and BMP receptor molecules. Altered binding affinities may in turn lead to differential activation of receptors that mediate different signaling pathways, which may ultimately lead to different biological activities or outcomes. Altered binding affinities could also be manifested in a tissue or cell type-specific manner, thereby inducing only particular progenitor cell types to undergo proliferation and/or differentiation.

[0077] In preferred embodiments, the pair of morphogenic polypeptides have amino acid sequences each comprising a sequence that shares a defined relationship with an amino acid sequence of a reference morphogen. Herein, preferred osteogenic polypeptides share a defined relationship with a sequence present in osteogenically

active human OP-1, SEQ ID NO: 1. However, any one or more of the naturally occurring or biosynthetic sequences disclosed herein similarly could be used as a reference sequence. Preferred osteogenic polypeptides share a defined relationship with at least the C-terminal six cysteine domain of human OP-1, residues 335-431 of SEQ ID NO: 1. Preferably, osteogenic polypeptides share a defined relationship with at least the C-terminal seven cysteine domain of human OP-1, residues 330-431 of SEQ ID NO: 1. That is, preferred polypeptides in a dimeric protein with bone morphogenic activity each comprise a sequence that corresponds to a reference sequence or is functionally equivalent thereto.

[0078] Functionally equivalent sequences include functionally equivalent arrangements of cysteine residues disposed within the reference sequence, including amino acid insertions or deletions which alter the linear arrangement of these cysteines, but do not materially impair their relationship in the folded structure of the dimeric morphogen protein, including their ability to form such intra- or inter-chain disulfide bonds as may be necessary for morphogenic activity. Functionally equivalent sequences further include those wherein one or more amino acid residues differs from the corresponding residue of a reference sequence, *e.g.*, the C-terminal seven cysteine domain (also referred to herein as the conserved seven cysteine skeleton) of human OP-1, provided that this difference does not destroy bone morphogenic activity. Accordingly, conservative substitutions of corresponding amino acids in the reference sequence are preferred. Amino acid residues that are conservative substitutions for corresponding residues in a reference sequence are those that are physically or functionally similar to the corresponding

reference residues, *e.g.*, that have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Particularly preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff *et al.*, *supra*, the teachings of which are incorporated by reference herein.

[0079] The osteogenic protein OP-1 has been described (see, *e.g.*, Oppermann *et al.*, U. S. Patent No. 5,354,557, incorporated herein by reference). Natural-sourced osteogenic protein in its mature, native form is a glycosylated dimer typically having an apparent molecular weight of about 30-36 kDa as determined by SDS-PAGE. When reduced, the 30 kDa protein gives rise to two glycosylated peptide subunits having apparent molecular weights of about 16 kDa and 18 kDa. The unglycosylated protein, which also has osteogenic activity, has an apparent molecular weight of about 27 kDa. When reduced, the 27 kDa protein gives rise to two unglycosylated polypeptides, having molecular weights of about 14 kDa to 16 kDa, capable of inducing endochondral bone formation in a mammal. Osteogenic proteins may include forms having varying glycosylation patterns, varying N-termini, and active truncated or mutated forms of native protein. As described above, particularly useful sequences include those comprising the C-terminal 96 or 102 amino acid sequences of DPP (from Drosophila), Vg1 (from Xenopus), Vgr-1 (from mouse), the OP-1 and OP-2 proteins,(see U.S. Pat. No. 5,011,691 and Oppermann *et al.*, incorporated herein by reference), as well as the proteins referred to as BMP-2, BMP-3, BMP-4 (see WO88/00205, U.S. Patent No. 5,013,649 and WO91/18098, incorporated herein by reference), BMP-5 and BMP-6 (see WO90/11366,

PCT/US90/01630, incorporated herein by reference), BMP-8 and BMP-9.

[0080] Preferred morphogenic and osteogenic proteins of this invention comprise at least one polypeptide selected from the group consisting of OP-1 (BMP-7), OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, TGF-β and amino acid sequence variants and homologs thereof, including species homologs, thereof. Preferably, the morphogenic protein comprises at least one polypeptide selected from the group consisting of OP-1 (BMP-7), BMP-2, BMP-4, BMP-5 and BMP-6; more preferably, OP-1 (BMP-7)and BMP-2; and most preferably, OP-1 (BMP-7).

[0081] Publications disclosing these sequences, as well as their chemical and physical properties, include: OP-1 and OP-2 (U.S. Patent No. 5,011,691; U.S. Patent No. 5,266,683; Ozkaynak *et al.*, EMBO J., 9, pp. 2085-2093 (1990); OP-3 (WO94/10203 (PCT US93/10520)), BMP-2, BMP-3, BMP-4, (WO88/00205; Wozney *et al.* Science, 242, pp. 1528-1534 (1988)), BMP-5 and BMP-6, (Celeste *et al.*, PNAS, 87, 9843-9847 (1991)), Vgr-1 (Lyons *et al.*, PNAS, 86, pp. 4554-4558 (1989)); DPP (Padgett *et al.* Nature, 325, pp. 81-84 (1987)); Vg-1 (Weeks, Cell, 51, pp. 861-867 (1987)); BMP-9 (WO95/33830 (PCT/US95/07084); BMP-10 (WO94/26893 (PCT/US94/05290); BMP-11 (WO94/26892 (PCT/US94/05288); BMP-12 (WO95/16035 (PCT/US94/14030); BMP-13 (WO95/16035 (PCT/US94/14030); GDF-1 (WO92/00382 (PCT/US91/04096) and Lee *et al.* PNAS, 88, pp. 4250-4254 (1991); GDF-8 (WO94/21681 (PCT/US94/03019); GDF-9

(WO94/15966 (PCT/US94/00685); GDF-10 (WO95/10539 (PCT/US94/11440); GDF-11 (WO96/01845 (PCT/US95/08543); BMP-15 (WO96/36710 (PCT/US96/06540); MP-121 (WO96/01316 (PCT/EP95/02552); GDF-5 (CDMP-1, MP52) (WO94/15949 (PCT/US94/00657) and WO96/14335 (PCT/US94/12814) and WO93/16099 (PCT/EP93/00350)); GDF-6 (CDMP-2, BMPl3) (WO95/01801 (PCT/US94/07762) and WO96/14335 and WO95/10635 (PCT/US94/14030)); GDF-7 (CDMP-3, BMP12). (WO95/10802 (PCT/US94/07799) and WO95/10635 (PCT/US94/14030)) The above publications are incorporated herein by reference. In another embodiment, useful proteins include biologically active biosynthetic constructs, including novel biosynthetic morphogenic proteins and chimeric proteins designed using sequences from two or more known morphogens.

[0082] In another embodiment of this invention, a morphogenic protein may be prepared synthetically to induce tissue formation. Morphogenic proteins prepared synthetically may be native, or may be non-native proteins, i.e., those not otherwise found in nature. Non-native osteogenic proteins have been synthesized using a series of consensus DNA sequences (U.S. Patent No. 5,324,819, incorporated herein by reference). These consensus sequences were designed based on partial amino acid sequence data obtained from natural osteogenic products and on their observed homologies with other genes reported in the literature having a presumed or demonstrated developmental function.

[0083] Several of the biosynthetic consensus sequences (called consensus osteogenic proteins or "COPs") have been expressed as fusion proteins in prokaryotes. Purified fusion proteins may be cleaved, refolded, implanted in an established animal model and shown to have bone- and/or cartilage-inducing activity. The

currently preferred synthetic osteogenic proteins comprise two synthetic amino acid sequences designated COP-5 (SEQ. ID NO: 2) and COP-7 (SEQ. ID NO: 3)

[0084] Oppermann *et al.*, U. S. Patent Nos. 5,011,691 and 5,324,819, which are incorporated herein by reference, describe the amino acid sequences of COP-5 and COP-7 as shown below:

COP5 LYVDFS-DVGW**D**DWIVAPPGY**Q**AFYCHGECPFPLAD
COP7 LYVDFS-DVGW**N**DWIVAPPGY**H**AFYCHGECPFPLAD

COP5 H**F**NSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
COP7 H**L**NSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA

COP5 ISMLYLDENEKVVLKYNQEMVVEGCGCR
COP7 ISMLYLDENEKVVLKYNQEMVVEGCGCR

[0085] In these amino acid sequences, the dashes (-) are used as fillers only to line up comparable sequences in related proteins. Differences between the aligned amino acid sequences are highlighted.

[0086] The DNA and amino acid sequences of these and other BMP family members are published and may be used by those of skill in the art to determine whether a newly identified protein belongs to the BMP family. New BMP-related gene products are expected by analogy to possess at least one morphogenic activity and thus classified as a BMP.

[0087] In one preferred embodiment of this invention, the morphogenic protein comprises a pair of subunits disulfide bonded to produce a dimeric species, wherein at least one of the subunits comprises a recombinant peptide belonging to the BMP protein family. In another preferred embodiment of this invention, the morphogenic

protein comprises a pair of subunits that produce a dimeric species formed through non-covalent interactions, wherein at least one of the subunits comprises a recombinant peptide belonging to the BMP protein family. Non-covalent interactions include Van der Waals, hydrogen bond, hydrophobic and electrostatic interactions. The dimeric species may be a homodimer or heterodimer and is capable of inducing cell proliferation and/or tissue formation.

[0088] In certain preferred embodiments, bone morphogenic proteins useful herein include those in which the amino acid sequences comprise a sequence sharing at least 70% amino acid sequence homology or "similarity", and preferably 80% homology or similarity, with a reference morphogenic protein selected from the foregoing naturally occurring proteins. Preferably, the reference protein is human OP-1, and the reference sequence thereof is the C-terminal seven cysteine domain present in osteogenically active forms of human OP-1, residues 330-431 of SEQ ID NO: 1. In certain embodiments, a polypeptide suspected of being functionally equivalent to a reference morphogen polypeptide is aligned therewith using the method of Needleman, *et al.*, *supra*, implemented conveniently by computer programs such as the Align program (DNAstar, Inc.). As noted above, internal gaps and amino acid insertions in the candidate sequence are ignored for purposes of calculating the defined relationship, conventionally expressed as a level of amino acid sequence homology or identity, between the candidate and reference sequences. In a currently preferred embodiment, the reference sequence is OP-1. Bone morphogenic proteins useful herein accordingly include allelic, phylogenetic counterpart and other variants of the preferred reference sequence, whether

naturally-occurring or biosynthetically produced (*e.g.*, including "muteins" or "mutant proteins"), as well as novel members of the general morphogenic family of proteins, including those set forth and identified above. Certain particularly preferred morphogenic polypeptides share at least 60% amino acid identity with the preferred reference sequence of human OP-1, still more preferably at least 65% amino acid identity therewith.

[0089] In another embodiment, useful osteogenic proteins include those sharing the conserved seven cysteine domain and sharing at least 70% amino acid sequence homology (similarity) within the C-terminal active domain, as defined herein. In still another embodiment, the osteogenic proteins of the invention can be defined as osteogenically active proteins having any one of the generic sequences defined herein, including OPX (SEQ ID NO: 4) and Generic Sequences 7 (SEQ ID NO: 5) and 8 (SEQ ID NO: 6), or Generic Sequences 9 (SEQ ID NO: 7) and 10 (SEQ ID NO: 8).

[0090] The family of bone morphogenic polypeptides useful in the present invention, and members thereof, can be defined by a generic amino acid sequence. For example, Generic Sequence 7 (SEQ ID NO: 5) and Generic Sequence 8 (SEQ ID NO: 6) are 97 and 102 amino acid sequences, respectively, and accommodate the homologies shared among preferred protein family members identified to date, including at least OP-1, OP-2, OP-3, CBMP-2A, CBMP-2B, BMP-3, 60A, DPP, Vg1, BMP-5, BMP-6, Vgr-1, and GDF-1. The amino acid sequences for these proteins are described herein and/or in the art, as summarized above. The generic sequences include both the amino acid identity shared by these sequences in the C-terminal domain, defined by the six and seven cysteine skeletons (Generic Sequences 7 and 8, respectively), as well as

alternative residues for the variable positions within the sequence. The generic sequences provide an appropriate cysteine skeleton where inter- or intramolecular disulfide bonds can form, and contain certain critical amino acids likely to influence the tertiary structure of the folded proteins. In addition, the generic sequences allow for an additional cysteine at position 36 (Generic Sequence 7) or position 41 (Generic Sequence 8), thereby encompassing the morphogenically active sequences of OP-2 and OP-3.

Generic Sequence 7

			Leu 1	Xaa	Xaa	Xaa	Phe 5	Xaa	Xaa
Xaa	Gly	Trp 10	Xaa	Xaa	Xaa	Xaa	Xaa 15	Xaa	Pro
Xaa	Xaa	Xaa 20	Xaa	Ala	Xaa	Tyr	Cys 25	Xaa	Gly
Xaa	Cys	Xaa 30	Xaa	Pro	Xaa	Xaa	Xaa 35	Xaa	Xaa
Xaa	Xaa	Xaa 40	Asn	His	Ala	Xaa	Xaa 45	Xaa	Xaa
Xaa	Xaa	Xaa 50	Xaa	Xaa	Xaa	Xaa	Xaa 55	Xaa	Xaa
Xaa	Xaa	Xaa 60	Cys	Cys	Xaa	Pro	Xaa 65	Xaa	Xaa
Xaa	Xaa	Xaa 70	Xaa	Xaa	Leu	Xaa	Xaa 75	Xaa	Xaa
Xaa	Xaa	Xaa 80	Val	Xaa	Leu	Xaa	Xaa 85	Xaa	Xaa
Xaa	Met	Xaa 90	Val	Xaa	Xaa	Cys	Xaa 95	Cys	Xaa

[0091]

wherein each Xaa independently is selected from a group of one or more specified amino acids defined as follows: "res." means "residue" and Xaa at res.2 = (Tyr or Lys); Xaa at res.3 = Val or Ile); Xaa at res.4 = (Ser, Asp or Glu); Xaa at res.6 = (Arg, Gln, Ser, Lys or Ala); Xaa at

res.7 = (Asp or Glu); Xaa at res.8 = (Leu, Val or Ile); Xaa at res. 11 = (Gln, Leu, Asp, His, Asn or Ser); Xaa at res.12 = (Asp, Arg, Asn or Glu); Xaa at res.13 = (Trp or Ser); Xaa at res.14 = (Ile or Val); Xaa at res.15 = (Ile or Val); Xaa at res.16 (Ala or Ser); Xaa at res.18 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res.19 = (Gly or Ser); Xaa at res.20 = (Tyr or Phe); Xaa at res.21 = (Ala, Ser, Asp, Met, His, Gln, Leu or Gly); Xaa at res.23 = (Tyr, Asn or Phe); Xaa at res.26 = (Glu, His, Tyr, Asp, Gln, Ala or Ser); Xaa at res.28 = (Glu, Lys, Asp, Gln or Ala); Xaa at res.30 = (Ala, Ser, Pro, Gln, Ile or Asn); Xaa at res.31 = (Phe, Leu or Tyr); Xaa at res.33 = (Leu, Val or Met); Xaa at res.34 = (Asn, Asp, Ala, Thr or Pro); Xaa at res.35 = (Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res.36 = (Tyr, Cys, His, Ser or Ile); Xaa at res.37 = (Met, Phe, Gly or Leu); Xaa at res.38 = (Asn, Ser or Lys); Xaa at res.39 = (Ala, Ser, Gly or Pro); Xaa at res.40 = (Thr, Leu or Ser); Xaa at res.44 = (Ile, Val or Thr); Xaa at res.45 = (Val, Leu, Met or Ile); Xaa at res.46 = (Gln or Arg); Xaa at res.47 = (Thr, Ala or Ser); Xaa at res.48 = (Leu or Ile); Xaa at res.49 = (Val or Met); Xaa at res.50 = (His, Asn or Arg); Xaa at res.51 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.52 = (Ile, Met, Asn, Ala, Val, Gly or Leu); Xaa at res.53 = (Asn, Lys, Ala, Glu, Gly or Phe); Xaa at res.54 = (Pro, Ser or Val); Xaa at res.55 = (Glu, Asp, Asn, Gly, Val, Pro or Lys); Xaa at res.56 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser, Gly, Ile or His); Xaa at res.57 = (Val, Ala or Ile); Xaa at res.58 = (Pro or Asp); Xaa at res.59 = (Lys, Leu or Glu); Xaa at res.60 = (Pro, Val or Ala); Xaa at res.63 = (Ala or Val); Xaa at res.65 = (Thr, Ala or Glu); Xaa at res.66 = (Gln, Lys, Arg or Glu); Xaa at res.67 = (Leu, Met or Val); Xaa at res.68 = (Asn, Ser, Asp or Gly); Xaa at res.69 =(Ala, Pro or Ser); Xaa at res.70 =

(Ile, Thr, Val or Leu); Xaa at res.71 = (Ser, Ala or Pro); Xaa at res.72 = (Val, Leu, Met or Ile); Xaa at res.74 = (Tyr or Phe); Xaa at res.75 = (Phe, Tyr, Leu or His); Xaa at res.76 = (Asp, Asn or Leu); Xaa at res.77 = (Asp, Glu, Asn, Arg or Ser); Xaa at res.78 = (Ser, Gln, Asn, Tyr or Asp); Xaa at res.79 = (Ser, Asn, Asp, Glu or Lys); Xaa at res.80 = (Asn, Thr or Lys); Xaa at res.82 = (Ile, Val or Asn); Xaa at res.84 = (Lys or Arg); Xaa at res.85 = (Lys, Asn, Gln, His, Arg or Val); Xaa at res.86 = (Tyr, Glu or His); Xaa at res.87 = (Arg, Gln, Glu or Pro); Xaa at res.88 = (Asn, Glu, Trp or Asp); Xaa at res.90 = (Val, Thr, Ala or Ile); Xaa at res.92 = (Arg, Lys, Val, Asp, Gln or Glu); Xaa at res.93 = (Ala, Gly, Glu or Ser); Xaa at res.95 = (Gly or Ala) and Xaa at res.97 = (His or Arg).

[0092] Generic Sequence 8 (SEQ ID NO: 6) includes all of Generic Sequence 7 and in addition includes the following sequence (SEQ ID NO: 9) at its N-terminus:

SEQ ID NO: 9

Cys	Xaa	Xaa	Xaa	Xaa
1				5

Accordingly, beginning with residue 7, each "Xaa" in Generic Sequence 8 is a specified amino acid defined as for Generic Sequence 7, with the distinction that each residue number described for Generic Sequence 7 is shifted by five in Generic Sequence 8. Thus, "Xaa at res.2 =(Tyr or Lys)" in Generic Sequence 7 refers to Xaa at res. 7 in Generic Sequence 8. In Generic Sequence 8, Xaa at res.2 = (Lys, Arg, Ala or Gln); Xaa at res.3 = (Lys, Arg or Met); Xaa at res.4 = (His, Arg or Gln); and Xaa at res. 5 = (Glu, Ser, His, Gly, Arg, Pro, Thr, or Tyr).

[0093] In another embodiment, useful osteogenic proteins include those defined by Generic Sequences 9 and 10, defined as follows.

[0094] Specifically, Generic Sequences 9 and 10 are composite amino acid sequences of the following proteins: human OP-1, human OP-2, human OP-3, human BMP-2, human BMP-3, human BMP-4, human BMP-5, human BMP-6, human BMP-8, human BMP-9, human BMP 10, human BMP-11, Drosophila 60A, Xenopus Vg-1, sea urchin UNIVIN, human CDMP-1 (mouse GDF-5), human CDMP-2 (mouse GDF-6, human BMP-13), human CDMP-3 (mouse GDF-7, human BMP-12), mouse GDF-3, human GDF-1, mouse GDF-1, chicken DORSALIN, dpp, Drosophila SCREW, mouse NODAL, mouse GDF-8, human GDF-8, mouse GDF-9, mouse GDF-10, human GDF-11, mouse GDF-11, human BMP-15, and rat BMP3b. Like Generic Sequence 7, Generic Sequence 9 is a 97 amino acid sequence that accommodates the C-terminal six cysteine skeleton and, like Generic Sequence 8, Generic Sequence 10 is a 102 amino acid sequence which accommodates the seven cysteine skeleton.

Generic Sequence 9

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10
Xaa Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa
15 20
Xaa Xaa Xaa Xaa Cys Xaa Gly Xaa Cys Xaa
25 30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
45 50
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
55 60
Xaa Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa
65 70
Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa
75 80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
85 90
Xaa Xaa Xaa Cys Xaa Cys Xaa
95

[0095]

wherein each Xaa is independently selected from a group of one or more specified amino acids defined as follows: "res." means "residue" and Xaa at res. 1 = (Phe, Leu or Glu); Xaa at res. 2 = (Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu); Xaa at res. 3 = (Val, Ile, Leu or Asp); Xaa at res. 4 = (Ser, Asp, Glu, Asn or Phe); Xaa at res. 5 = (Phe or Glu); Xaa at res. 6 = (Arg, Gln, Lys, Ser, Glu, Ala or Asn); Xaa at res. 7 = (Asp, Glu, Leu, Ala or Gln); Xaa at res. 8 = (Leu, Val, Met, Ile or Phe); Xaa at res. 9 = (Gly, His or Lys); Xaa at res. 10 = (Trp or Met); Xaa at res. 11 = (Gln, Leu, His, Glu, Asn, Asp, Ser or Gly); Xaa at res. 12 = (Asp, Asn, Ser, Lys, Arg, Glu or His); Xaa at res. 13 = (Trp or Ser); Xaa at res. 14 = (Ile or Val); Xaa at res. 15 = (Ile or Val); Xaa at res. 16 = (Ala, Ser, Tyr or Trp); Xaa at res. 18 = (Glu, Lys, Gln, Met, Pro, Leu, Arg, His or Lys); Xaa at res. 19 = (Gly, Glu, Asp, Lys, Ser, Gln, Arg or Phe); Xaa at res. 20 = (Tyr or Phe); Xaa at res. 21 = (Ala, Ser, Gly, Met, Gln, His, Glu, Asp, Leu, Asn, Lys or Thr); Xaa at res. 22 = (Ala or Pro); Xaa at res. 23 = (Tyr, Phe, Asn, Ala or Arg); Xaa at res. 24 = (Tyr, His, Glu, Phe or Arg); Xaa at res. 26 = (Glu, Asp, Ala, Ser, Tyr, His, Lys, Arg, Gln or Gly); Xaa at res. 28 = (Glu, Asp, Leu, Val, Lys, Gly, Thr, Ala or Gln); Xaa at res. 30 = (Ala, Ser, Ile, Asn, Pro, Glu, Asp, Phe, Gln or Leu); Xaa at res. 31= (Phe, Tyr, Leu, Asn, Gly or Arg); Xaa at res. 32 = (Pro, Ser, Ala or Val); Xaa at res. 33 = (Leu, Met, Glu, Phe or Val); Xaa at res. 34 = (Asn, Asp, Thr, Gly, Ala, Arg, Leu or Pro); Xaa at res. 35 = (Ser, Ala, Glu, Asp, Thr, Leu,

Lys, Gln or His); Xaa at res. 36 = (Tyr, His, Cys, Ile, Arg, Asp, Asn, Lys, Ser, Glu or Gly); Xaa at res. 37 = (Met, Leu, Phe, Val, Gly or Tyr); Xaa at res. 38 = (Asn, Glu, Thr, Pro, Lys, His, Gly, Met, Val or Arg); Xaa at res. 39 = (Ala, Ser, Gly, Pro or Phe); Xaa at res. 40 = (Thr, Ser, Leu, Pro, His or Met); Xaa at res. 41 = (Asn, Lys, Val, Thr or Gln); Xaa at res. 42 = (His, Tyr or Lys); Xaa at res. 43 = (Ala, Thr, Leu or Tyr); Xaa at res. 44 = (Ile, Thr, Val, Phe, Tyr, Met or Pro); Xaa at res. 45 = (Val, Leu, Met, Ile or His); Xaa at res. 46 = (Gln, Arg or Thr); Xaa at res. 47 = (Thr, Ser, Ala, Asn or His); Xaa at res. 48 = (Leu, Asn or Ile); Xaa at res. 49 = (Val, Met, Leu, Pro or Ile); Xaa at res. 50 = (His, Asn, Arg, Lys, Tyr or Gln); Xaa at res. 51 = (Phe, Leu, Ser, Asn, Met, Ala, Arg, Glu, Gly or Gln); Xaa at res. 52 = (Ile, Met, Leu, Val, Lys, Gln, Ala or Tyr); Xaa at res. 53 = (Asn, Phe, Lys, Glu, Asp, Ala, Gln, Gly, Leu or Val); Xaa at res. 54 = (Pro, Asn, Ser, Val or Asp); Xaa at res. 55 = (Glu, Asp, Asn, Lys, Arg, Ser, Gly, Thr, Gln, Pro or His); Xaa at res. 56 = (Thr, His, Tyr, Ala, Ile, Lys, Asp, Ser, Gly or Arg); Xaa at res. 57 = (Val, Ile, Thr, Ala, Leu or Ser); Xaa at res. 58 = (Pro, Gly, Ser, Asp or Ala); Xaa at res. 59 = (Lys, Leu, Pro, Ala, Ser, Glu, Arg or Gly); Xaa at res. 60 = (Pro, Ala, Val, Thr or Ser); Xaa at res. 61 = (Cys, Val or Ser); Xaa at res. 63 = (Ala, Val or Thr); Xaa at res. 65 = (Thr, Ala, Glu, Val, Gly, Asp or Tyr); Xaa at res. 66 = (Gln, Lys, Glu, Arg or Val); Xaa at res. 67 = (Leu, Met, Thr or Tyr); Xaa at res. 68 = (Asn, Ser, Gly, Thr, Asp, Glu, Lys or Val); Xaa at res. 69 = (Ala, Pro, Gly or Ser); Xaa at res. 70 = (Ile, Thr, Leu or Val); Xaa at res. 71 = (Ser, Pro, Ala, Thr, Asn or Gly); Xaa at res. 2 = (Val, Ile, Leu or Met); Xaa at res. 74 = (Tyr, Phe, Arg, Thr, Tyr or Met); Xaa at res. 75 = (Phe, Tyr, His, Leu, Ile, Lys, Gln

or Val); Xaa at res. 76 = (Asp, Leu, Asn or Glu); Xaa at res. 77 = (Asp, Ser, Arg, Asn, Glu, Ala, Lys, Gly or Pro); Xaa at res. 78 = (Ser, Asn, Asp, Tyr, Ala, Gly, Gln, Met, Glu, Asn or Lys); Xaa at res. 79 = (Ser, Asn, Glu, Asp, Val, Lys, Gly, Gln or Arg); Xaa at res. 80 = (Asn, Lys, Thr, Pro, Val, Ile, Arg, Ser or Gln); Xaa at res. 81 = (Val, Ile, Thr or Ala); Xaa at res. 82 = (Ile, Asn, Val, Leu, Tyr, Asp or Ala); Xaa at res. 83 = (Leu, Tyr, Lys or Ile); Xaa at res. 84 = (Lys, Arg, Asn, Tyr, Phe, Thr, Glu or Gly); Xaa at res. 85 = (Lys, Arg, His, Gln, Asn, Glu or Val); Xaa at res. 86 = (Tyr, His, Glu or Ile); Xaa at res. 87 = (Arg, Glu, Gln, Pro or Lys); Xaa at res. 88 = (Asn, Asp, Ala, Glu, Gly or Lys); Xaa at res. 89 = (Met or Ala); Xaa at res. 90 = (Val, Ile, Ala, Thr, Ser or Lys); Xaa at res 91 = (Val or Ala); Xaa at res. 92 = (Arg, Lys, Gln, Asp, Glu, Val, Ala, Ser or Thr); Xaa at res. 93 = (Ala, Ser, Glu, Gly, Arg or Thr); Xaa at res. 95 = (Gly, Ala or Thr); Xaa at res. 97 = (His, Arg, Gly, Leu or Ser). Further, after res. 53 in rBMP3b and mGDF-10 there is an Ile; after res. 54 in GDF-1 there is a T ; after res. 54 in BMP3 there is a V; after res. 78 in BMP-8 and Dorsalin there is a G; after res. 37 in hGDF-1 there is Pro, Gly, Gly, Pro.

[0096] Generic Sequence 10 (SEQ ID NO: 8) includes all of Generic Sequence 9 (SEQ ID NO: 7) and in addition includes the following sequence (SEQ ID NO: 9) at its N-terminus:

SEQ ID NO: 9

Cys	Xaa	Xaa	Xaa	Xaa
1				5

Accordingly, beginning with residue 6, each "Xaa" in Generic Sequence 10 is a specified amino acid defined as for Generic Sequence 9, with the distinction that each

residue number described for Generic Sequence 9 is shifted by five in Generic Sequence 10. Thus, "Xaa at res. 1 = (Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu)" in Generic Sequence 9 refers to Xaa at res. 6 in Generic Sequence 10. In Generic Sequence 10, Xaa at res. 2 = (Lys, Arg, Gln, Ser, His, Glu, Ala, or Cys); Xaa at res. 3 = (Lys, Arg, Met, Lys, Thr, Leu, Tyr, or Ala); Xaa at res. 4 = (His, Gln, Arg, Lys, Thr, Leu, Val, Pro, or Tyr); and Xaa at res. 5 = (Gln, Thr, His, Arg, Pro, Ser, Ala, Gln, Asn, Tyr, Lys, Asp, or Leu).

[0097] As noted above, certain currently preferred bone morphogenic polypeptide sequences useful in this invention have greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the preferred reference sequence of hOP-1. These particularly preferred sequences include allelic and phylogenetic counterpart variants of the OP-1 and OP-2 proteins, including the Drosophila 60A protein. Accordingly, in certain particularly preferred embodiments, useful morphogenic proteins include active proteins comprising pairs of polypeptide chains within the generic amino acid sequence herein referred to as "OPX" (SEQ ID NO: 4), which defines the seven cysteine skeleton and accommodates the homologies between several identified variants of OP-1 and OP-2. As described therein, each Xaa at a given position independently is selected from the residues occurring at the corresponding position in the C-terminal sequence of mouse or human OP-1 or OP-2.

SEQ ID NO: 4

```
Cys Xaa Xaa His Glu Leu Tyr Val Ser Phe Xaa Asp Leu Gly Trp Xaa Asp Trp
1               5                  10                  15
Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly Glu Cys Xaa Phe Pro
    20                  25                  30                  35
Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala Ile Xaa Gln Xaa Leu Val His Xaa
        40                  45                  50                  55
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Xaa Xaa Pro Xaa Xaa Val Pro Lys Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala
60 65 70
Xaa Ser Val Leu Tyr Xaa Asp Xaa Ser Xaa Asn Val Ile Leu Xaa Lys Xaa Arg
75 80 85 90
Asn Met Val Val Xaa Ala Cys Gly Cys His
95 100

[0098]

wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 11 = (Arg or Gln); Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr); Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr or His); and Xaa at res. 97 = (Arg or Lys).

[0099] In still another preferred embodiment, useful osteogenically active proteins have polypeptide chains with amino acid sequences comprising a sequence encoded by a nucleic acid that hybridizes, under low, medium or high stringency hybridization conditions, to DNA or RNA encoding reference morphogen sequences, *e.g.*, C-terminal sequences defining the conserved seven cysteine domains of OP-1, OP-2, BMP-2, BMP-4, BMP-5, BMP-6, 60A, GDF-3, GDF-6, GDF-7 and the like. As used herein, high stringent hybridization conditions are defined as hybridization according to known techniques in 40% formamide, 5 X SSPE, 5 X Denhardt's Solution, and 0.1%

SDS at 37°C overnight, and washing in 0.1 X SSPE, 0.1% SDS at 50°C. Standard stringent conditions are well characterized in commercially available, standard molecular cloning texts. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D.N. Glover ed., 1985); Oligonucleotide Synthesis (M.J. Gait ed., 1984): Nucleic Acid Hybridization (B. D. Hames & S.J. Higgins eds. 1984); and B. Perbal, A Practical Guide To Molecular Cloning (1984), the disclosures of which are incorporated herein by reference.

[0100] As noted above, proteins useful in the present invention generally are dimeric proteins comprising a folded pair of the above polypeptides. Such morphogenic proteins are inactive when reduced, but are active as oxidized homodimers and when oxidized in combination with others of this invention to produce heterodimers. Thus, members of a folded pair of morphogenic polypeptides in a morphogenically active protein can be selected independently from any of the specific polypeptides mentioned above.

[0101] The bone morphogenic proteins useful in the materials and methods of this invention include proteins comprising any of the polypeptide chains described above, whether isolated from naturally-occurring sources, or produced by recombinant DNA or other synthetic techniques, and includes allelic and phylogenetic counterpart variants of these proteins, as well as muteins thereof, and various truncated and fusion constructs. Deletion or addition mutants also are envisioned to be active, including those which may alter the conserved C-terminal six or seven cysteine domain, provided that the alteration does not functionally

disrupt the relationship of these cysteines in the folded structure. Accordingly, such active forms are considered the equivalent of the specifically described constructs disclosed herein. The proteins may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native or biosynthetic proteins, produced by expression of recombinant DNA in host cells.

[0102] The bone morphogenic proteins contemplated herein can be expressed from intact or truncated cDNA or from synthetic DNAs in prokaryotic or eukaryotic host cells, and purified, cleaved, refolded, and dimerized to form morphogenically active compositions. Currently preferred host cells include, without limitation, prokaryotes including *E. coli* or eukaryotes including yeast, or mammalian cells, such as CHO, COS or BSC cells. One of ordinary skill in the art will appreciate that other host cells can be used to advantage. Detailed descriptions of the bone morphogenic proteins useful in the practice of this invention, including how to make, use and test them for osteogenic activity, are disclosed in numerous publications, including U.S. Patent Nos. 5,266,683 and 5,011,691, the disclosures of which are incorporated by reference herein.

[0103] Thus, in view of this disclosure and the knowledge available in the art, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different biological species, which encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both prokaryotes and eukaryotes, to produce large quantities of active

proteins capable of stimulating endochondral bone morphogenesis in a mammal.

Implant Device

[0104] The invention also relates to an implant device for promoting bone formation, regeneration and repair. The implant device comprises the bone precursor composition material of the invention, and optionally at least one additional agent selected from a bioactive agent or a binder.

[0105] The implant device comprising a cement matrix formed from the bone precursor composition serves as a temporary scaffold and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation.

[0106] In a preferred embodiment, the implant device comprises the bone precursor composition and a bioactive agent, which is dispersed or absorbed in the bone precursor composition. The cement matrix formed from the bone precursor composition provides a delivery or support system for the bioactive agent, which is released over time at the implantation site as the bone precursor composition is slowly absorbed. In a preferred embodiment, the bioactive agent is encapsulated in the pore-forming agent. The resorption of the pore-forming agent and the gradual release of the bioactive agent provides a sustained release system. The dosage and rate of delivery of the bioactive agent may be controlled based on the nature of the cement, the nature of the pore-forming agent and the nature of the binding interaction between the bioactive agent, the cement and pore-forming agent. In a preferred embodiment, the bioactive agent is a bone morphogenic protein or a

nucleic acid molecule that encodes BMP. In a more preferred embodiment, the BMP is OP-1.

[0107] The cement matrix formed by the bone precursor composition can protect the BMP from non-specific proteolysis, and can accommodate each step of the cellular responses involved in progenitor cell induction during tissue development.

[0108] In the preferred osteogenic device with the cement matrix formed by the bone precursor composition, the osteogenic protein diffuses out of the cement matrix into the implantation site and permits influx and efflux of cells. The osteogenic protein induces the progenitor cells to differentiate and proliferate. Progenitor cells may migrate into the cement matrix and differentiated cells may move out of the cement matrix into the implant site. The sequential cellular reactions in the interface of the cement matrix/osteogenic protein implants include: binding of fibrin and fibronectin to implanted cement matrix, migration and proliferation of mesenchymal cells, differentiation of the progenitor cells into chondroblasts, cartilage formation, cartilage calcification, vascular invasion, bone formation, remodeling, and bone marrow differentiation. The preferred osteogenic device with the bone precursor composition, can be used in various orthopedic, periodontal, and reconstructive procedures.

[0109] The implant device may also comprise a binder in an admixture with the bioactive agent and/or bone precursor composition material. The binder is added to regulate the moldability of the composition to fit a defect site or to take the form of a new tissue. In one embodiment, the implant material may be molded into a variety of shapes depending on the tissue defect site to which it is administered. In another embodiment, the

implant material may be injected. For example, healing of closed fresh fractures can be accelerated through single minimally invasive percutaneous injections of implanted material. See, *e.g.*, Blokhuis *et al.* Biomaterials 22, pp. 725-730 (2001), incorporated herein by reference. In yet another embodiment, the implant material may have a preferred shape which allows implantation at a defect site. The moldable cement matrix formed from the bone precursor composition can be held in place by the surrounding tissue or masticated muscle. It is preferred to shape the composition to span a tissue defect and to take the desired form of the new tissue. In the case of bone repair of a non-union defect, for example, it is desirable to use dimensions that span the non-union. Rat studies show that the newly synthesized bone has the dimensions of the implanted device. Thus, the material may be used for subcutaneous or intramuscular implants. In bone formation procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

Prosthetic Device

[0110] It is also contemplated that the bone precursor composition material of the present invention may be used in a prosthetic device. The prosthetic device comprises a surface region that can be implanted adjacent to a target tissue of a mammal, and a composition that is disposed on the surface region. The prosthetic devices will be useful for repairing orthopedic defects, injuries or anomalies in the treated mammal. Preferably, the mammal is a human patient. The prosthetic device may be made from a material comprising metal, ceramic or polymer composite material. Preferred devices comprise a load-

bearing core selected from Co-Cr-Mo alloys, titanium alloys and stainless steel. Preferred prosthetic devices are selected from the group consisting of a hip device, a fusion cage and a maxillofacial device.

[0111] The composition comprises the bone precursor composition of the invention, and optionally, one or more agents selected from the group consisting of a bioactive agent or a binder dispersed in the bone precursor composition. In a preferred embodiment, the bioactive agent is encapsulated in the pore-forming agent. In another preferred embodiment, the bioactive agent is a BMP or nucleic acid comprising a sequence encoding BMP, more preferably, OP-1.

[0112] The composition may act as a coating for synthetically constructed bone material, such as for an artificial hip, replacement of diseased bone, correction of defects, or anchoring teeth. Osteogenic protein-coated prosthetic devices may enhance the bond strength between the prosthesis and existing bone (Rueger *et al.*, U. S. Patent No. 5,344,654, incorporated herein by reference). In a preferred embodiment, the prosthetic device is coated with a hydroxyapatite or beta-tricalcium phosphate material to facilitate the integration of the composition of this invention onto the prosthetic device. This embodiment is particularly advantageous when there is a lack of bone mass around the prosthetic device. The composition is disposed on the surface of the implant in an amount sufficient to promote enhanced tissue growth into the surface. The amount of the composition sufficient to promote enhanced tissue growth may be determined empirically by those of skilled in the art using bioassays described in Rueger *et al.*, U. S. patent No. 5,344,654, incorporated herein by reference. Preferably, animal studies are performed to optimize the

concentration of the composition components before a similar prosthetic device is used in the human patient.

[0113] In a preferred embodiment, the composition can be used in ligament repair such as anterior cruciate ligament fixation or ligament attachment in the appendicular system to assist in the integration of ligament and bone.

[0114] In another preferred embodiment, the composition is applied to the clinical procedure of total joint arthroplasty in hips, knees, elbows and other joints, wherein a diseased or damaged natural joint is replaced by a prosthetic joint. For example, in a total hip arthroplasty, an acetabular cup is inserted with the composition in the acetabular socket of the pelvis to replace the natural acetabulum. The cup is held in place by the composition and secured by fixation screws. Generally, the cavity or socket conforms to the outer surface of the acetabular cup. The composition can also be applied to total joint revision surgery, to strengthen the bondage between joint prosthetic devices and the bone.

[0115] In yet another preferred embodiment, the composition is applied to a clinical procedure called vertebroplasty. The composition is injected into the interior of a vertebral body. This method is used in the treatment of osteoporosis to increase the density of bone.

[0116] In a preferred embodiment, the prosthetic device is selected from the group consisting of a fusion cage, a dowel and other devices having a pocket or chamber, such as an interbody fusion for containing the composition of the present invention. Preferably, the interbody fusion device is produced from material selected from the group consisting of titanium, PEEK

(poly(etheretherketone)) and allograft. The interbody fusion in the cervical, thoracic and lumbar spine can be administered via an anterior or posterior approach. Alternatively, the composition of this invention can be used without an associated interbody device to achieve interbody fusion.

[0117] Spinal fusion cages are placed into the intervertebral space left after the removal of a damaged spinal disc to eliminate local motion and to participate in vertebral to vertebra bony fusion. As described in U.S. patent No. 5,015,247, the fusion cages are in the form of a cylindrical hollow member having an outside diameter larger than the space between two adjacent vertebrae to be fused. The interior space within the cylindrical hollow implant can be filled with the composition of this invention. The cylindrical implants can also include a threaded exterior to permit threaded insertion into a tapped bore formed in the adjacent vertebrae. Alternatively, some fusion implants have been designed to be impacted into the intradiscal space. As described in U.S. patent No. 6,146,420, the fusion device includes opposite end pieces with an integral central element. The central element has a much smaller diameter so that the fusion device forms an annular pocket around the central element. The composition of this invention can be disposed within the annular pocket between the opposite end pieces.

[0118] In a preferred embodiment, the prosthetic device is used for repair of osseous and discoligamentous instability. The composition of this invention may be applied to the intervertebral area, resulting in superior fusion and consequently achieving definitive stabilization of a traumatized motor segment via a single dorsal approach. This application may eliminate the need

to undergo a second operation for fractures of the thoracolumbar spine, which, at present, is often necessary but involves additional high risks. Also, this method avoids the problems associated with transplantation of autogenous cancellous bone and its associated risk of high morbidity. See, *e.g.*, Rueger *et al.*, Orthopäde, 27, pp. 72-79 (1998).

[0119] In another preferred embodiment, the prosthetic device is a maxillofacial device. Maxillofacial devices are applied externally to correct facial defects resulting from cancer surgery, accidents, congenital deformities. In order to restore the masticatory deficiencies, a patient with marginal bone mass is first treated with the composition of this invention to pack and build up the surgical site. A maxillofacial anchoring and distracting system, as illustrated in U.S. patent No. 5,899,940, can be applied to increase the existing bone quality. Fixation devices, such as a standard threaded bone screw and simple pin point tack or self-locking and threaded bone tack screw device (U.S. patent No. 5,971,985), are used for the retention of tissue grafts and synthetic membranes to the maxillofacial bone graft site. Once the site has healed, a second surgery is performed to insert the appropriate length endosseous dental implant and to restore masticatory function.

[0120] The invention also provides a method for promoting *in vivo* integration of an implantable prosthetic device of this invention into a target tissue of a mammal comprising the steps of a) providing on a surface of the prosthetic device a composition comprising the bone precursor composition, optionally, at least one bioactive agent or a binder, and b) implanting the device in a mammal at a locus where the target tissue and the

surface of the prosthetic device are maintained at least partially in contact for a time sufficient to permit tissue growth between the target tissue and the device.

Method of Inducing Bone Formation and Delivery

[0121] The invention also provides a method of inducing bone formation or repair in a mammal. The mammal is preferably a human patient. The method comprises the step of implanting in the defect site of a mammal a composition comprising the bone precursor composition of the invention. In a preferred embodiment, the composition may further comprise a binder and/or a bioactive agent. Preferably, the bioactive agent is encapsulated in the pore-forming agent. The defect can be an endochondral defect, an osteochondral defect or a segmental defect. The method can also be applied to other defects which include, but are not limited to, non-union fractures; bone cavities; tumor resection; fresh fractures (distracted or undistracted); cranial, maxillofacial and facial abnormalities, for example, in facial skeletal reconstruction, specifically, orbital floor reconstruction, augmentation of the alveolar ridge or sinus, periodontal defects and tooth extraction socket; cranioplasty, genioplasty, chin augmentation, palate reconstruction, and other large bony reconstructions; vertebroplasty, interbody fusions in the cervical, thoracic and lumbar spine and posteriolateral fusions in the thoracic and lumbar spine; in osteomyelitis for bone regeneration; appendicular fusion, ankle fusion, total hip, knee and joint fusions or arthroplasty; correcting tendon and/or ligamentous tissue defects such as, for example, the anterior, posterior, lateral and medial ligaments of the knee, the patella and achilles tendons, and the like as well as those defects

resulting from diseases such as cancer, arthritis, including osteoarthritis, and other bone degenerative disorders such as osteochondritis dessicans.

[0122] The invention also provides a method of delivering a bioactive agent at a site requiring bone formation comprising the step of implanting the bone precursor composition and a bioactive agent at the defect site of a mammal. The method of delivering the bone precursor composition may further include a binder. In a preferred embodiment, the bioactive agent is encapsulated in the pore-forming agent. In a preferred embodiment, the bioactive agent belongs to the bone morphogenic protein family. In another preferred embodiment, the bioactive agent is a nucleic acid molecule comprising a sequence encoding a BMP. Preferably, the nucleic acid is trapped in a carrier. In yet another embodiment, the bioactive agent is a bone cell or a bone cell expressing BMP. In another preferred embodiment, the delivery of the bioactive agent is sustained release. The pore-forming agent is preferably a biocompatible and non-immunogenic polymer, more preferably, PLGA. The bioactive agent is preferably OP-1. The release rate of the bioactive agent can be controlled by altering the molecular weight of the PLGA. The degradation of PLGA commences when water penetrates the cement matrix to hydrolyze long polymer chains into short water soluble fragments. There is a reduction in molecular weight of the PLGA without loss in its physical properties. Gradually, further erosion of the polymer leads to the disruption of the polymer, thereby releasing the bioactive agent. For example, in the case of 10 kD to 30 kD PLGA, the rate of release for OP-1 is one to six weeks.

Examples

EXAMPLE 1: Formation of Hydroxyapatite Implants with Pore-forming Agents

[0123] The PLGA polymer microspheres were supplied from Alkermes, Inc. The calcium sulfate pore-forming agent was prepared by hydrating calcium sulfate hemihydrate. The wet calcium sulfate mass was passed through a sieve to form granules, which were hardened after drying, and resieved to break any agglomerates.

[0124] Microsphere PLGA beads having a particle size of 75-150 μm were mixed with BoneSource® hydroxyapatite cement powder (containing tetracalcium phosphate and dicalcium phosphate anhydrous) at different ratios (Table 1).

TABLE 1

PLGA (%)	Hydroxyapatite Cement Powder (mg)	PLGA (mg)	H_2O (ml)	Setting time (min)
0	2000	0	500	27
10	1800	200	560	22
20	1600	400	560	19
30	1400	600	560	23
50	1000	1000	560	29

[0125] Calcium sulfate granules having a particle size of 75-500 μm were mixed with BoneSource® hydroxyapatite cement powder at different ratios (Table 2).

TABLE 2

$CaSO_4$ (%)	Hydroxyapatite Cement Powder (mg)	$CaSO_4$ (mg)	H_2O (ml)
0	2000	0	500
10	1800	200	500
20	1600	400	500
30	1400	600	530
50	1000	1000	530

[0126] The two cement mixtures described above were kept at 37°C in a high humidity incubator for 24 hours to dry and harden.

EXAMPLE 2: Hardness Testing of Cement Mixtures

[0127] In both the PLGA and $CaSO_4$ implants, the bulk density of the implants decreased as the percentage of hydroxyapatite decreased. The decrease in bulk density was more pronounced for the PLGA incorporated implants (Table 3) than in the calcium sulfate incorporated implants (Table 4). Bulk density determination was performed by measuring the mass and volume of the implant material. The lightness of the PLGA material may have lead to the pronounced reduction in bulk density.

TABLE 3

PLGA (%)	Bulk Density (g/cc)	Compression strength (Mpa)
0	1.96 ± 0.02	25.56 ± 3.70
10	1.76 ± 0.01	24.21 ± 2.64
20	1.59 ± 0.02	19.55 ± 2.33
30	1.43 ± 0.01	15.39 ± 1.57
50	1.06 ± 0.03	4.77 ± 0.19

TABLE 4

CaSO4 (%)	Bulk Density (g/cc)	Compression strength (Mpa)
0	1.96 ± 0.02	25.56 ± 3.70
10	1.90 ± 0.02	18.95 ± 2.84
20	1.89 ± 0.03	13.90 ± 5.97
30	1.84 ± 0.06	15.30 ± 1.08
50	1.73 ± 0.06	8.91 ± 1.81

EXAMPLE 3: Simulation of *in vivo* Resorption Activity

[0128] The hardened implants were then treated with 0.2 N HCl acid for 24 hours to conduct a rapid simulation of the *in vivo* resorption activity. 5 ml of 0.2 N HCl was added to each implant that was placed in a 5 ml glass vial. The acid surface covered the implant completely. The vial was stoppered and placed on an automatic shaker with moderate shaking. The appearance of the implants was observed periodically.

[0129] After 7 hours, the structural rigidity of all the tested implants was intact. In both the calcium sulfate and PLGA incorporated implants, increased porosity was observed in direct proportion to the increase in pore-forming agent. However, the calcium sulfate implants were observed to be more brittle as they held structural rigidity after 24 hours of acid treatment. The 100% hydroxyapatite implant did not show any visible porosity. The implants with pore-forming agents showed varying degrees of porosity. The implants containing 50% pore-forming agents were visibly very porous while maintaining their structure. Calcium sulfate implants developed larger and more visible pores than the PLGA implants.

EXAMPLE 4: Rat Model Bioassay for Bone Induction

[0130] This assay consists of implanting the composition of this invention in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, may be used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants are removed at varying times thereafter (i.e. 12 days, 18 days). The heterotrophic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotropic sites.

[0131] Bone growth is determined biochemically by calcium content of the implant. Calcium content, is proportional to the amount of bone formed in the implant. Bone formation therefore is calculated by determining the calcium content of the implant in rats and is expressed as "bone forming units," where one bone forming unit represents the amount of protein that is needed for half maximal bone forming activity of the implant. Bone induction exhibited by intact demineralized rat bone matrix is considered to be the maximal bone differentiation activity for comparison purposes in this assay.

Cellular Events During Endochondral Bone Formation

[0132] Successful implants exhibit a controlled progression through the stages of protein-induced endochondral bone development, including: (1) transient infiltration by polymorphonuclear leukocytes; (2)

mesenchymal cell migration and proliferation; (3) chondrocyte appearance; (4) cartilage matrix formation; (5) cartilage calcification; (6) vascular invasion, appearance of osteoblasts, and formation of new bone; (7) appearance of osteoclasts, bone remodeling and dissolution of the implanted matrix; and (8) hematopoietic bone marrow differentiation in the ossicles. This time course in rats may be accelerated by increasing the amounts of OP-1 added. The shape of the new bone conforms to the shape of the implanted cement matrix.

Histological evaluation

[0133] Histological sectioning and staining is a preferred method to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8 μm sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve-day implants are usually sufficient to determine whether the implants contain newly-induced bone.

Biological markers

[0134] Alkaline phosphatase (AP)activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity *in vivo* peaks subsequently and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantification and obtaining an estimate of bone formation quickly after the implants are removed from the rat. Alternatively, the amount of bone

formation can be determined by measuring the calcium content of the implant.

[0135] Gene expression patterns that correlate with endochondral bone or other types of tissue formation can also be monitored by quantitating mRNA levels using procedures known to those of skill in the art such as Northern Blot analysis. Such developmental gene expression markers may be used to determine progression through tissue differentiation pathways after treatment with osteogenic protein. These markers include osteoblastic-related matrix proteins such as procollagen α_2 (I), procollagen α_1(I), procollagen α_1(III), osteonectin, osteopontin, biglycan, and alkaline phosphatase for bone regeneration (see *e.g.*, Suva *et al.*, J. Bone Miner. Res., 8, pp. 379-88 (1993); Benayahu *et al.*, J. Cell. Biochem., 56, pp. 62-73 (1994)).

EXAMPLE 5: Sheep Model Bioassay for Bone Repair

[0136] Three drilled defects were created in the area of the proximal metaphysis for both the left and right tibia of skeletally mature female sheep. Defects were 6 mm in diameter and at least 10 mm deep. The defect size was consistent across all test animals. The defects were created so as to maintain the structure of the interosseous fibrofatty marrow. This marrow acts as a barrier between the implant materials and prevents interosseous mixing of the matrix materials tested. The different formulations tested are listed in Table 5. The cement used was from BoneSource®.

[0137] A 3 to 4 inch incision was made over the proximal tibial metaphysis. The skin and underlying muscle were dissected to expose the periosteum. The periosteum was incised and maintained intact for surgical closure if possible. Three transverse holes were created

in the metaphysis. The first and most superior was created approximately 2 cm below the articular surface of the tibia. The defects were created so as to form a line oriented with the long axis of the bone. Implants were spaced at 1.6 cm intervals measured center-to-center.

[0138] Materials were harvested at four and eight weeks post-treatment. Animals were euthanised with pentobarbital 75-100 mg/kg. The proximal tibia was taken and cut to best allow for tissue fixation. Specimens were fixed in 10% neutral buffered Formalin. Specimens were cut, if feasible, so as to capture all implant sites in a single specimen. Following fixation, specimens were decalcified, embedded in plastic and sectioned in longitudinal orientation using Exackt technique and ground to appropriate section thickness for histologic interpretation.

[0139] Radiographic assessment and histologic evaluation of all implant sites were made at four and eight weeks post-operative. Anterior posterior radiographs were taken so as to best image all three defects simultaneously and view the cylindrical defects from the side. Qualitative histologic descriptions identified new bone formation, residual implant material and any evidence of pathologic response. Images were captured for each specimen and scores presented for bone formation, acute and chronic inflammation and residual matrix.

[0140] Specimen handling and hemostatic properties were recorded at the time of implantation. Materials were in the form of a putty with good consistency. The cement matrix was implanted following the mixing of the formulation and saline solution.

TABLE 5

Formulation	Composition
1	Collagen, Placebo
2	cement, 14% (w/w) PLGA 10 kD
3	cement, 14% (w/w) PLGA 25-30 kD
6	Collagen, OP-1
7	cement, 14% (w/w) PLGA 10 kD with 0.15% (w/w) OP-1
8	cement, 14% (w/w) PLGA 25-30 kD with 0.15% (w/w) OP-1
9	cement, 7% (w/w) PLGA 10 kD with 0.30% (w/w) OP-1
10	cement, 7% (w/w) PLGA 25-30 kD with 0.30% (w/w) OP-1
control	untreated, no test sample

Histologic Results

[0141] Proximal tibia sections contained three defects. These sections were gross macro-cut so that all three defects were contained in a single section. Based on gross section observations, clinical assays, and faxitron x-rays of this section, the section was considered representative of the sample. This orientation allowed the evaluation of the periosteal reaction overlying the defects and intramedullary response to the test materials. Specimens were evaluated from 4 and 8 week explants (Figures 1-6).

Paraffin Histology Study

[0142] Tissues from the sheep model bioassay were evaluated for bone formation using paraffin sections and hematoxylin and eosin stain. Tibial specimens were

sectioned so as to isolate implant sites in the proximal, middle and distal sites from animals. These explants were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

[0143] Sections were viewed using light microscopy and interpreted for bone formation. For specimens stratified in bone formation, the response from the cortical level was robust and deep, and the response was modest in the medullary compartment. Due to this stratification, the level extending from the endosteal cortex to a level 2-3 mm deep was evaluated.

EXAMPLE 6: Feline Model Bioassay for Bone Repair

[0144] A femoral osteotomy defect is surgically prepared. Without further intervention, the simulated fracture defect would consistently progress to non-union. The effects of the composition and devices of this invention implanted into the created bone defects are evaluated by the following study protocol.

[0145] Briefly, the procedure is as follows: Sixteen adult cats each weighing less than 10 lbs. undergo unilateral preparation of a 1 cm bone defect in the right femur through a lateral surgical approach. In other experiments, a 2 cm bone defect may be created. The femur is immediately internally fixed by lateral placement of an 8-hole plate to preserve the exact dimensions of the defect. Three different types of materials may be implanted in the surgically created cat femoral defects: group I is a negative control group with no test material; group II is implanted with the cement matrix formed from the bone precursor composition; group III is implanted with the cement matrix formed from the bone precursor composition and an osteogenic protein.

[0146] All animals are allowed to ambulate *ad libitum*

within their cages post-operatively. All cats are injected with tetracycline (25 mg/kg subcutaneously (SQ) each week for four weeks) for bone labeling.

[0147] *In vivo* radiomorphometric studies are carried out immediately at 4, 8, 12 and 16 weeks post-operative by taking a standardized X-ray of the lightly-anesthetized animal positioned in a cushioned X-ray jig designed to consistently produce a true anterio-posterior view of the femur and the osteotomy site. All X-rays are taken in exactly the same fashion and in exactly the same position on each animal. Bone repair is calculated as a function of mineralization by means of random point analysis. A final specimen radiographic study of the excised bone is taken in two planes after sacrifice.

[0148] Excised test and normal femurs may be immediately studied by bone densitometry, or wrapped in two layers of saline-soaked towels, placed into sealed plastic bags, and stored at -20° C until further study. Bone repair strength, load-to-failure, and work-to-failure are tested by loading to failure on a specially designed steel 4-point bending jig attached to an Instron testing machine to quantitate bone strength, stiffness, energy absorbed and deformation to failure. The study of test femurs and normal femurs yields the bone strength (load) in pounds and work-to-failure in joules. Normal femurs exhibit a strength of 96 (+/- 12) pounds. Osteogenic device-implanted femur strength should be corrected for surface area at the site of fracture (due to the "hourglass" shape of the bone defect repair). With this correction, the result should correlate closely with normal bone strength.

[0149] Following biomechanical testing, the bones are immediately sliced into two longitudinal sections at the defect site, weighed, and the volume measured. One-half

is fixed for standard calcified bone histomorphometrics with fluorescent stain incorporation evaluation, and one-half is fixed for decalcified hemotoxylin/eosin stain histology preparation.

[0150] Selected specimens from the bone repair site are homogenized in cold 0.15 M NaCl, 3 mM $NaHCO_3$, pH 9.0 by a Spex freezer mill. The alkaline phosphatase activity of the supernatant and total calcium content of the acid soluble fraction of sediment are then determined.

EXAMPLE 7: Rabbit Model Bioassay for Bone Repair

[0151] This assay is described in detail in Oppermann *et al.*, U. S. Patent No. 5,354,557; see also Cook *et al.*, J. of Bone and Joint Surgery, 76-A, pp. 827-38 (1994), which are incorporated herein by reference). Ulnar non-union defects of 1.5 cm are created in mature (less than 10 lbs) New Zealand White rabbits with epiphyseal closure documented by X-ray. The experiment may include implantation of devices into at least eight rabbits per group as follows: group I is a negative control group with no test material; group II is implanted with the cement matrix formed from the bone precursor composition; and group III is implanted with the cement matrix formed from the bone precursor composition and an osteogenic protein. Ulnae defects are followed for the full course of the eight week study in each group of rabbits.

[0152] In another experiment, the marrow cavity of the 1.5 cm ulnar defect is packed with osteogenic protein in a cement matrix formed from the bone precursor composition. The bones are allografted in an intercalary fashion. Negative control ulnae are not healed by eight weeks and reveal the classic "ivory" appearance. In distinct contrast, the osteogenic protein-treated

implants "disappear" radiographically by four weeks with the start of remineralization by six to eight weeks. These allografts heal at each end with mild proliferative bone formation by eight weeks. This type of device serves to accelerate allograft repair.

[0153] As was described above, the rabbit model may also be used to test the efficacy of and to optimize conditions under which a particular composition of this invention can induce local bone formation.

EXAMPLE 8: Dog Ulnar Defect Bioassay For Bone Repair

[0154] This assay is performed essentially as described in Cook *et al.*, Clinical Orthopaedics and Related Research, 301, pp. 302-112 (1994), which is incorporated herein by reference). Briefly, an ulnar segmental defect model is used to evaluate bone healing in 35-45 kg adult male dogs. Experimental composites comprising 500 mg of bone precursor composition are reconstituted with varying amounts of OP-1. Any osteogenic protein may be used in place of OP-1 in this assay. Implantations at defect sites are performed with one carrier control and with the experimental series of OP-1 being tested. Mechanical testing is performed on ulnae of animals receiving composites at 12 weeks after implantation. Radiographs of the forelimbs are obtained weekly until the animals are sacrificed at either 12 or 16 postoperative weeks. Histological sections are analyzed from the defect site and from adjacent normal bone.

EXAMPLE 9: Monkey Ulnar and Tibial Defect Bioassay For Bone Repair

[0155] This bone healing assay in African green monkeys is performed essentially as described in Cook *et al.*, J. Bone and Joint Surgery, 77A, pp. 734-50 (1995), which is incorporated herein by reference. Briefly, a 2.0 cm osteoperiosteal defect is created in the middle of the ulnar shaft and filled with an implant comprising bone precursor composition matrices containing OP-1. Experimental composites comprising bone precursor composition matrices reconstituted with varying amounts of OP-1 are used to fill 2.0 cm osteoperiosteal defects created in the diaphysis of the tibia. Any osteogenic protein may be used in place of OP-1 in this assay. Implantations at defect sites are performed with one carrier control and with the experimental series of OP-1 being tested. Mechanical testing is performed on ulnae and tibia of animals receiving composites. Radiographs and histological sections are analyzed from the defect sites and from adjacent normal bone as described in Cook *et al.*

EXAMPLE 10: Goat Model Fracture Healing Bioassay

[0156] This fracture healing assay in sheep is performed essentially as described in Blokhius *et al.*, Biomaterials, 22, pp. 725-730 (2001), which is incorporated herein by reference. A closed midshaft fracture is created in the left tibia of adult female goats with a custom-made three point bending device. The fractures are stabilized with an external fixator, which is placed at the lateral side of the tibia. Three different types of materials are implanted in the goat defects via injection: group I is a negative control

group with no test material; group II is implanted with the cement matrix formed from the bone precursor composition; and group III is implanted with the cement matrix formed from the bone precursor composition and an osteogenic protein. The injections are given under aseptic conditions and fluroscopy is used to ascertain that the injected material was placed in the fracture gap. Mechanical testing (four-point non-destructive bending test) is performed on the animals receiving composites at two weeks and four weeks. After the mechanical testing, anterior, posterior, lateral, and medial slices of the fracture gap are sawn to perform radiographs and histological sections.

EXAMPLE 11: Fusion Assay of an Unstable Motor Segment of the Sheep Lumbar Spine

[0157] This assay investigates the healing of osseous and discoligamentous instability. A motor segment of the spine is a functional unit consisting of two vertebral bodies lying one above the other, and an intervertebral disc.

[0158] A trial group consists of 12 sheep. Two control groups of 12 sheep each are used. The surgical area at the inferior lumbar spine is prepared after introduction of general anesthesia and placing the animals in prone position. A skin incision of about 12 cm in length above the spinous processes of the inferior lumbar spine is made. After transsection of the subcutis and fascia, the back muscles are moved to the side.

[0159] Intubation anesthesia is applied by intramuscular injection of 1.5 ml xylazine (Rompun®). Further dosage can be administered as needed. The sedation requires placement of an intravenous indwelling

catheter after puncturing an ear vein. The anesthesia is introduced through the catheter by providing 3-5 mg of thiopental (Trapanal®) per killogram of body weight. After endotracheal intubation, the animals are ventilated using oxygen (30%), nitrous oxide (laughing gas) and isoflurane (Isofluran®). During the entire surgery, the analgesic fentanyl dihydrogen citrate (Fentanyl®) having a dosage 0.2-0.4 mg, is administered. At the same time, relaxation is achieved by administration of atracurium besilate (Atracurium®) at a dosage of 0.5mg/kg of body weight.

[0160] After complete exposure of the pedicles of lumbar vertebral bodies L4 to L6, a bilateral instrumentation of the pedicles L4 and L6 takes place. This is performed by using pedicle screws of 5 mm or 6 mm in diameter, depending on the diameter found in the pedicles. Subsequently, a bilateral transpedicular removal of the disc of the cranial motor segment L4/L5 is performed over the pedicle of L5 under pediculoscopic control. The endplates of the affected vertebral bodies will be decorticated.

[0161] Inter- and intracorporal application of test samples occurs via a transpedicular cannula in all 12 sheep of the trial group. Test samples include cement matrix and osteogenic protein in varying concentrations. In the first control group that consists of 12 sheep, only the cement matrix is applied. In the second control group, autologous spongiosa is administered instead of the composition of this invention.

[0162] Finally, the internal fixator is installed completely. The type of the internal fixator as well as the necessary instrumentation is the same as those used in humans. Accordingly, the surgical procedure is standardized and well known to the skilled practitioner.

Drains are placed and the wound is closed using absorbable suture for fascia and subcutis as well as skin staples.

[0163] During the entire surgical procedure, an x-ray image amplifier is available for intraoperative fluoroscopy. This facilitates exact orientation during the execution of the above steps.

[0164] Harvesting of the 12 sheep administered with autologous spongiosa is carried out under anesthesia as follows: the left iliac crest skin and fascia is cut by making a longitudinal incision about 8 cm long. The gluteal muscles are moved subperiostally and the cancellous bone graft is harvested from the iliac crest after an osteotomy. Hemostatis controlled excessive bleeding and placement of a drain is performed upon closure of the wound in layers. The harvesting procedure is standard and known to an ordinary person skilled in the art.

Clinical Observations

[0165] Daily neurologic examinations are performed to evaluate the gait of the animals as well as neurological deficits that may occur postoperatively. Operative wounds are closely examined each day. Body weights are measured preoperatively and at the time of euthanasia.

Radiographic Analysis

[0166] Before evaluation, the complete lumbar spine is freshly dissected, and the internal fixator is carefully removed. Anteroposterior and plain lateral radiographs of the operated spinal segments are obtained under consistent conditions of milliamperes, kilovolts, and seconds at 0 and 8 weeks to assist in fusion

evaluation. The status of the fusion will be evaluated with use of the grading system documented by Lenke *et al.*, J. Spinal Disord, 5, pp. 433-442 (1992), incorporated herein by reference. With this system, A indicates a big, solid trabeculated bilateral fusion mass (definitely solid); B, a big, solid unilateral fusion mass with a small contralateral fusion mass (possibly solid); C, a small, thin bilateral fusion mass with an apparent crack (probably not solid); and D, bilateral resorption of the graft or fusion mass with an obvious bilateral pseudarthrosis (definitely not solid).

[0167] Additionally, computerized tomography scans are performed to assess the fusion mass in cross sections and in saggital-plane reconstructions. For each fusion mass, approximately forty sequential computerized tomography scans are made with use of two-millimeter slice intervals and subsequent reconstruction in the saggital plane under consistent magnification and radiographic conditions.

Biomechanical Testing

[0168] Four specimens of each group are evaluated biomechanically. After radiographic analysis, all muscles are carefully removed while maintaining the ligamentous and bony structures. The spines are frozen at -20°C. For each of these specimens, the upper half of the upper vertebra and the lower half of the lower vertebra of the motion segment L4/L5 are embedded in polymethylmethacrylate (Technovit 3040; Heraeus Kulzer GmbH, Wehrheim/Ts, Germany). Each specimen is then fixed and tested without preload in a spine tester in a non-destructive testing mode. Alternating sequences of flexion/extension, axial right/left rotation, and right/left lateral bending moments are applied continuously at a constant rate of 1.7 degrees/second by

stepper motors integrated in the gimbal of the spine tester. Two precycles are applied to minimize the effect of the viscous component in the viscoelastic response, and data will be collected on the third cyle. Range of motion, neutral zone, and two stiffness parameters are determined from the resulting load-deformation curves.

Histology/Histomorphometry

[0169] Eight specimens of each group are evaluated histologically after two, four or eight weeks postoperatively. After radiographic analysis, the spines are fixed in 10% formalin-solution. Cross sections of either specimen are obtained to evaluate bony fusion, cellular reactions, biocompatibility, and signs of cement-integration/degradation. Qualitative histologic assessment of the fusion mass at the operative site are made for the presence of giant cells, inflammatory cells, or fibrous responses where the implanted materials may have been encapsulated. In addition, the osteoid found within the trabecular fusion mass and the amount of trabecular bone are assessed. Histomorphometric variables, such as the percentage of osteoid, osteoid thickness, number of osteoblasts per millimeter bone surface, and number of osteoclasts per millimeter bone surface are determined.

Fluorochrome Labeling

[0170] Eight animals are subjected to intravenuous application of 90 milligrams of xylenol orange per kilogram of body weight two weeks postoperatively, 10 milligrams of calcein green per kilogram of body weight four weeks postoperatively, and 25 milligrams of doxycyclinhyclate yellow per kilogram of body weight six

weeks postoperatively. This regimen follows the method published by Rahn and Perren. See, *e.g.*, Rahn *et al.*, Stain Technology, 46, pp. 125-129 (1971); Rahn *et al.*, Akt Traumatol, 10, pp. 109-115 (1980). Fluorochrome sequential analysis is then performed by Fluorescence microscopy on the specimens under UV light for qualitative and quantitative dynamic evaluation.

[0171] While we have described a number of embodiments of this invention, it is apparent that our basic constructions may be altered to provide other embodiments which utilize the products and processes of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the appended claims, rather than by the specific embodiments which have been presented by way of example.

We claim:

1. A bone precursor composition comprising a cement mixture and a pore-forming agent, wherein the pore-forming agent has a particle size of 20-500 μm; with the proviso that when the pore-forming agent is PLGA, the particle size is 20-140 μm or 310-500 μm and when the pore forming agent is calcium sulfate, the particle size is 20-140 μm or 260-500 μm.

2. A bone precursor composition comprising a cement mixture and a pore-forming agent, wherein the pore-forming agent has a particle size of 20-140 μm.

3. A bone precursor composition comprising a cement mixture and a pore-forming agent, wherein the pore-forming agent has a particle size of 75-140 μm.

4. A bone precursor composition according to any one of claims 1 to 3, wherein the proportion of pore-forming agent is 7-40% (w/w).

5. A bone precursor composition according to any one of claims 1 to 3, wherein the proportion of pore-forming agent is 7-25% (w/w).

6. A bone precursor composition according to any one of claims 1 to 3, wherein the proportion of pore-forming agent is 7-14% (w/w) and the pore-forming agent is PLGA.

7. The bone precursor composition of any one of claims 1 to 3, wherein the cement mixture comprises a mixture selected from the group consisting of a calcium

phosphate cement mixture and a calcium sulfate cement mixture.

8. The bone precursor composition of any one of claims 1 to 3, wherein the cement mixture comprises a mixture selected from the group consisting of:

a) a mixture of decarbonated amorphous calcium phosphate and a second calcium phosphate;

b) a mixture of tetracalcium phosphate and a second calcium phosphate;

c) a mixture of monocalcium phosphate, tricalcium phosphate and calcium carbonate;

d) a mixture of beta-tricalcium phosphate and monocalcium phosphate monohydrate, and optionally, comprising calcium pyrophosphate, calcium sulfate dihydrate and calcium sulfate hemihydrate;

e) a mixture of beta-tricalcium phosphate, dicalcium phosphate dihydrate and calcium carbonate; and

f) calcium sulfate hemihydrate.

9. The bone precursor composition of claim 8, wherein the second calcium phosphate is selected from the group consisting of monocalcium phosphate, dicalcium phosphate anhydrous, dicalcium phosphate dihydrate, calcium metaphosphate, heptacalcium phosphate, calcium pyrophosphate, alpha-tricalcium phosphate, beta-tricalcium phosphate, octacalcium phosphate and amorphous calcium phosphate.

10. The bone precursor composition of any one of claims 1 to 3, wherein the pore-forming agent is a natural or synthetic biodegradable polymer.

11. The bone precursor composition of any one of claims 1 to 3, wherein the pore-forming agent is selected from the group consisting of ethylenevinylacetate, natural and synthetic collagen, poly(glaxanone), poly(phosphazenes), polyglactin, polyglactic acid, polyaldonic acid, polyacrylic acids, polyalkanoates, polyorthoesters, poly(L-lactide) (PLLA), poly(D,L-lactide) (PDLLA), polyglycolide (PGA), poly(lactide-co-glycolide (PLGA), poly(ζ-caprolactone), poly(trimethylene carbonate), poly(p-dioxanone), poly(ζ-caprolactone-co-glycolide), poly(glycolide-co-trimethylene carbonate) poly(D,L-lactide-co-trimethylene carbonate), polyarylates, polyhydroxybutyrate (PHB), polyanhydrides, poly(anhydride-co-imide) and co-polymers thereof, polymers of amino acids, propylene-co-fumarates, a polymer of one or more α-hydroxy carboxylic acid monomers, calcium sulfate, bioactive glass compositions, admixtures thereof and any derivatives and modifications thereof; with the proviso that when the cement mixture is calcium sulfate hemihydrate, the pore-forming agent is not calcium sulfate.

12. The bone precursor composition of claim 11, wherein the PLGA has a molecular weight of 5 kD to 100 kD.

13. The bone precursor composition of claim 11, wherein the PLGA has a molecular weight of 10 kD to 30 kD.

14. The bone precursor composition of any one of claims 1 to 3, wherein the cement mixture comprises a mixture of tetracalcium phosphate and dicalcium phosphate

anyhydrous; and the pore-forming agent is selected from the group consisting of PLGA and calcium sulfate.

15. The bone precursor composition of any one of claims 1 to 3, wherein the cement mixture comprises calcium sulfate hemihydrate, and the pore-forming agent is PLGA.

16. The bone precursor composition of any one of claims 1 to 3, further comprising a bioactive agent.

17. The bone precursor composition of claim 16, wherein the bioactive agent is a bone morphogenic protein.

18. The bone precursor composition of claim 16 wherein the bioactive agent is a nucleic acid molecule comprising a sequence encoding a bone morphogenic protein.

19. The bone precursor composition of claim 17, wherein the bone morphogenic protein is selected from the group consisting of OP-1, OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, TGF-β and conservative amino acid sequence variants thereof having osteogenic activity.

20. The bone precursor composition of claim 16, wherein the bioactive agent is an osteogenic protein

comprising an amino acid sequence having at least 70% homology with the C-terminal 102-106 amino acids of human OP-1.

21. The bone precursor composition of claim 16, wherein the bioactive agent is encapsulated in the pore-forming agent.

22. The bone precursor composition according to any one of claims 1 to 3 further comprising a binder.

23. The bone precursor composition of claim 22, wherein the binder is selected from the group consisting of sodium alginate, hyaluronic acid, sodium hyaluronate, gelatin, peptides, mucin, chrondroitin sulfate, chitosan, poloxamer, glycosaminoglycan, polysaccharide, polyethylene glycol, methylcellulose, carboxy methylcellulose, carboxy methylcellulose sodium, carboxy methylcellulose calcium, hydroxypropyl methylcellulose, hydroxybutyl methylcellulose, hydroxyethyl methylcellulose, hydroxyethylcellulose, methylhydroxyethyl cellulose, hydroxyethyl cellulose, mannitol, white petrolatum, mannitol/dextran combinations, mannitol/white petrolatum combinations, sesame oil, fibrin glue, blood and admixtures thereof.

24. The bone precursor composition of any one of claims 1 to 3 that is implantable.

25. A composition comprising a solid cement and a pore-forming agent, wherein the pore-forming agent has a particle size of 20-500 μm; with the proviso that when the pore-forming agent is PLGA, the particle size is 20-140 μm or 310-500 μm and when the pore forming agent

is calcium sulfate, the particle size is 20-140 μm or 260-500 μm.

26. A composition comprising a solid cement and a pore-forming agent, wherein the pore-forming agent has a particle size of 20-140 μm.

27. A composition comprising a solid cement and a pore-forming agent, wherein the pore-forming agent has a particle size of 75-140 μm.

28. The composition of any one of claims 25 to 27, wherein the proportion of pore-forming agent is 7-40% (w/w).

29. The composition of any one of claims 25 to 27, wherein the proportion of pore-forming agent is 7-25% (w/w).

30. The composition of any one of claims 25 to 27, wherein the pore-forming agent is PLGA and the proportion is 7-14% (w/w).

31. The composition of any one of claims 25 to 27, wherein the solid cement comprises a cement selected from the group consisting of a calcium phosphate cement and a calcium sulfate cement.

32. The composition of any one of claims 25 to 27, wherein the pore-forming agent is a natural or synthetic biodegradable polymer.

33. The composition of any one of claims 25 to 27, wherein the pore-forming agent is selected from the

group consisting of ethylenevinylacetate, natural and synthetic collagen, poly(glaxanone), poly(phosphazenes), polyglactin, polyglactic acid, polyaldonic acid, polyacrylic acids, polyalkanoates, polyorthoesters, poly(L-lactide) (PLLA), poly(D,L-lactide) (PDLLA), polyglycolide (PGA), poly(lactide-co-glycolide (PLGA), poly(ζ-caprolactone), poly(trimethylene carbonate), poly(p-dioxanone), poly(ζ-caprolactone-co-glycolide), poly(glycolide-co-trimethylene carbonate) poly(D,L-lactide-co-trimethylene carbonate), polyarylates, polyhydroxybutyrate (PHB), polyanhydrides, poly(anhydride-co-imide) and co-polymers thereof, polymers of amino acids, propylene-co-fumarates, a polymer of one or more α-hydroxy carboxylic acid monomers, calcium sulfate, bioactive glass compositions, admixtures thereof and any derivatives and modifications thereof; with the proviso that when the cement mixture is calcium sulfate hemihydrate, the pore-forming agent is not calcium sulfate.

34. The composition of claim 33, wherein the PLGA has a molecular weight of 5 kD to 100 kD.

35. The composition of claim 33, wherein the PLGA has a molecular weight of 10 kD to 30 kD.

36. A kit comprising:

a) the bone precursor composition of any one of claims 1 to 3; and

b) a bioactive agent.

37. The kit of claim 36, wherein the bioactive agent is a bone morphogenic protein.

38. The kit of claim 36, wherein the bioactive agent is a nucleic acid molecule comprising a sequence encoding a bone morphogenic protein.

39. The kit of claim 37, wherein the bone morphogenic protein is selected from the group consisting of OP-1, OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, TGF-β and conservative amino acid sequence variants thereof having osteogenic activity.

40. The kit of claim 36, wherein the bioactive agent is an osteogenic protein comprising an amino acid sequence having at least 70% homology with the C-terminal 102-106 amino acids of human OP-1.

41. A kit comprising:

a) the bone precursor composition of any one of claims 1 to 3; and

b) a binder.

42. The kit of claim 41, wherein the binder is selected from the group consisting of sodium alginate, hyaluronic acid, sodium hyaluronate, gelatin, peptides, mucin, chrondroitin sulfate, chitosan, poloxamer, glycosaminoglycan, polysaccharide, polyethylene glycol, methylcellulose, carboxy methylcellulose, carboxy methylcellulose sodium, carboxy methylcellulose calcium, hydroxypropyl methylcellulose, hydroxybutyl methylcellulose, hydroxyethyl methylcellulose,

hydroxyethylcellulose, methylhydroxyethyl cellulose, hydroxyethyl cellulose, mannitol, white petrolatum, mannitol/dextran combinations, mannitol/white petrolatum combinations, sesame oil, fibrin glue, blood and admixtures thereof.

43. An implantable prosthetic device comprising:

a) a prosthetic implant having a surface region implantable adjacent to a target tissue; and

b) the bone precursor composition of any one of claims 1 to 3 disposed on the surface region.

44. The prosthetic device of claim 43 further comprising a bioactive agent dispersed in the bone precursor composition.

45. The prosthetic device of claim 44, wherein the bioactive agent is a bone morphogenic protein.

46. The prosthetic device of claim 44, wherein the bioactive agent is a nucleic acid molecule comprising a sequence encoding a bone morphogenic protein.

47. The prosthetic device of claim 45, wherein the bone morphogenic protein is selected from the group consisting of OP-1, OP-2, OP-3, COP-1, COP-3, COP-4, COP-5, COP-7, COP-16, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP-16, BMP-17, BMP-18, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, MP121, dorsalin-1, DPP, Vg-1, Vgr-1, 60A protein, NODAL, UNIVIN, SCREW, ADMP, NEURAL, TGF-β and

conservative amino acid sequence variants thereof having osteogenic activity.

48. The prosthetic device of claim 44, wherein the bioactive agent is an osteogenic protein comprising an amino acid sequence having at least 70% homology with the C-terminal 102-106 amino acids of human OP-1.

49. The prosthetic device of claim 43, wherein the device is selected from the group consisting of a hip device, a fusion cage and a maxillofacial device.

50. The prosthetic device of claim 43, wherein the bioactive agent is encapsulated in the pore-forming agent.

51. The prosthetic device of claim 43 further comprising a binder.

52. The prosthetic device of claim 51, wherein the binder is selected from the group consisting of sodium alginate, hyaluronic acid, sodium hyaluronate, gelatin, peptides, mucin, chrondroitin sulfate, chitosan, poloxamer, glycosaminoglycan, polysaccharide, polyethylene glycol, methylcellulose, carboxy methylcellulose, carboxy methylcellulose sodium, carboxy methylcellulose calcium, hydroxypropyl methylcellulose, hydroxybutyl methylcellulose, hydroxyethyl methylcellulose, hydroxyethylcellulose, methylhydroxyethyl cellulose, hydroxyethyl cellulose, mannitol, white petrolatum, mannitol/dextran combinations, mannitol/white petrolatum combinations, sesame oil, fibrin glue, blood and admixtures thereof.

53. A method of inducing bone formation in a mammal comprising the step of implanting in the defect site of said mammal a composition comprising the bone precursor composition according to any one of claims 1 to 3.

54. The method of claim 53, wherein the composition further comprises a bioactive agent.

55. The method of claim 54, wherein the bioactive agent is a bone morphogenic protein.

56. The method of claim 53, wherein the composition further comprises a binder.

57. A method of delivering a bioactive agent at a site requiring bone formation comprising implanting at the defect site of a mammal a composition comprising the bone precursor composition of claims 1 to 3 and a bioactive agent.

58. The method of claim 57, wherein the bioactive agent is a bone morphogenic protein.

59. The method of claim 57, wherein the bioactive agent is encapsulated in the pore-forming agent.

60. The method of claim 59, wherein the delivery of the bioactive agent is sustained released.

61. The method of claim 57, wherein the bioactive agent is a nucleic acid molecule comprising a sequence encoding a bone morphogenic protein.

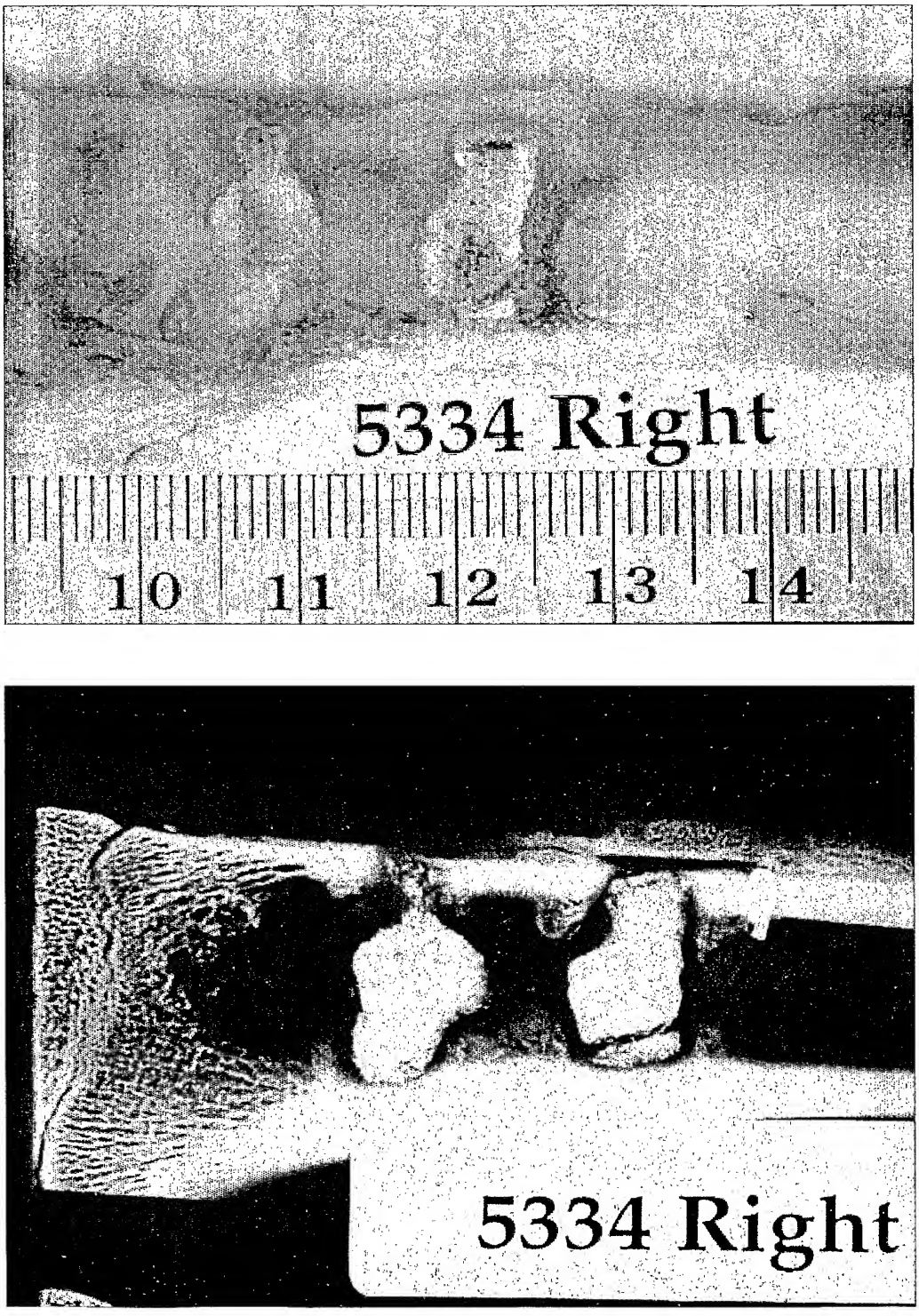
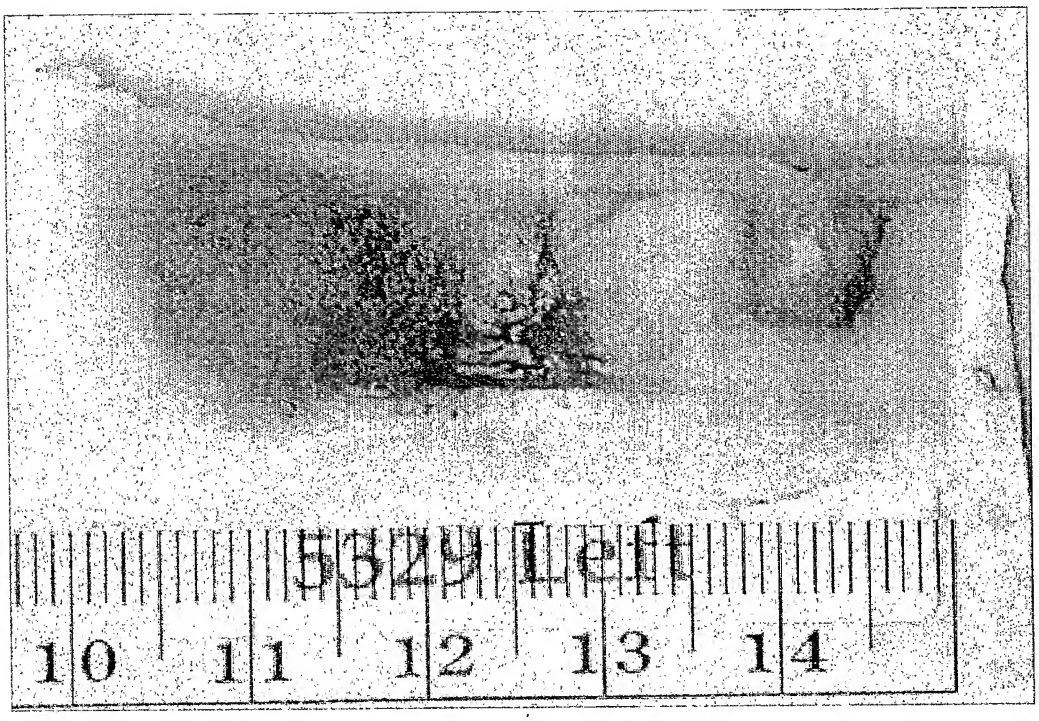
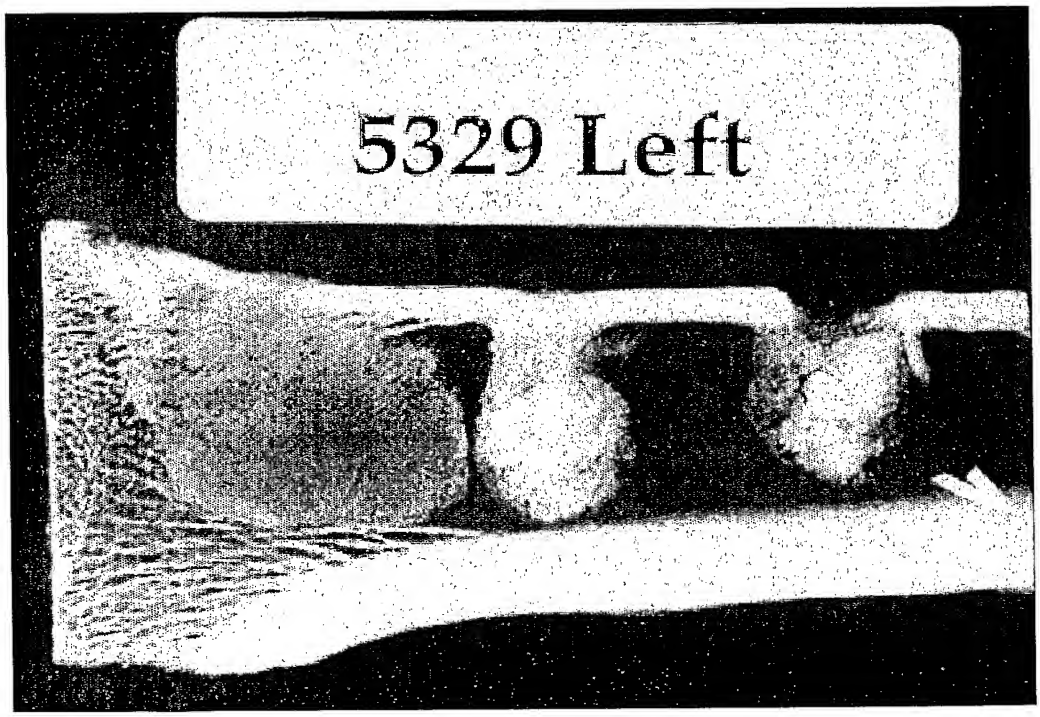


Figure 1





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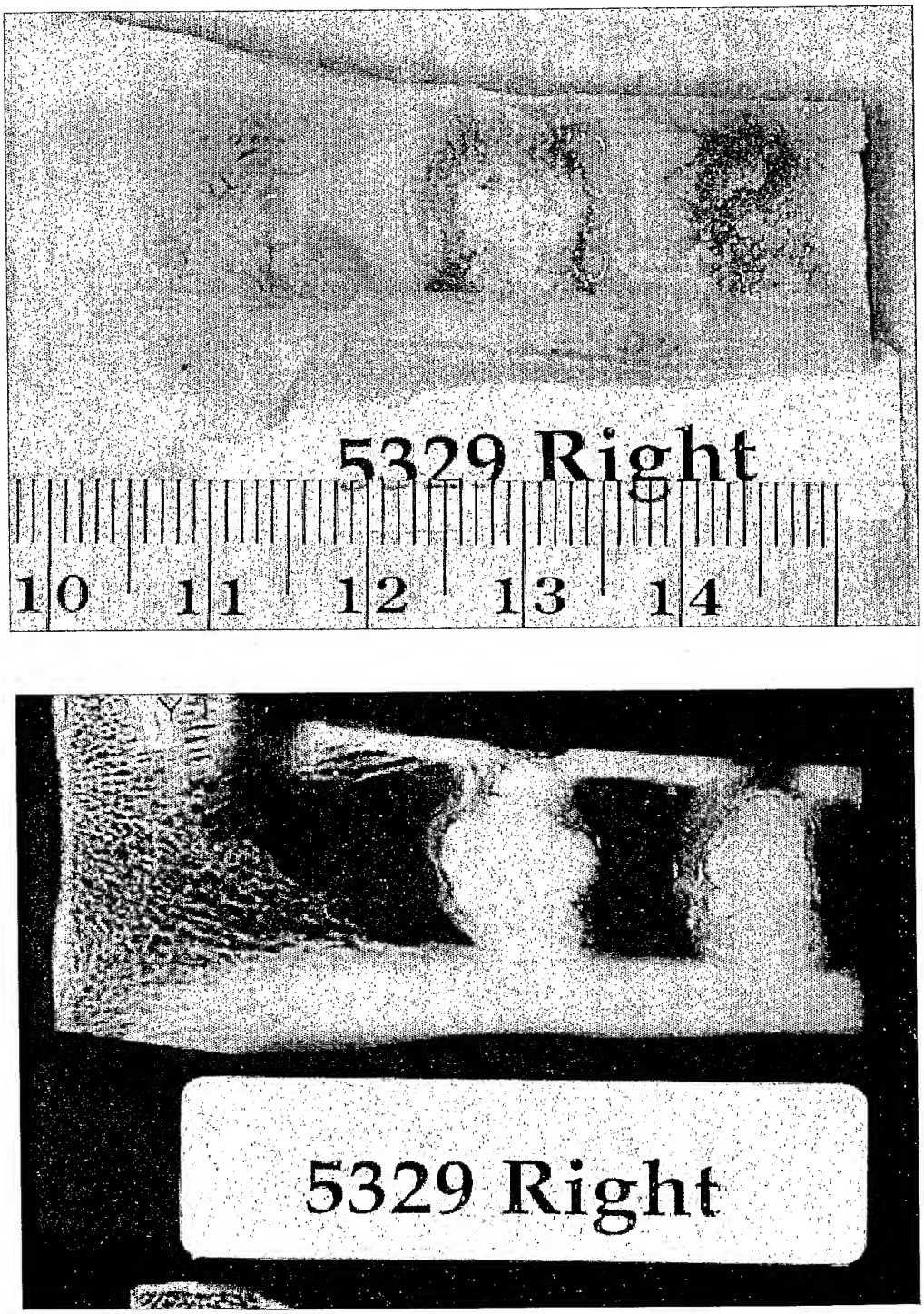

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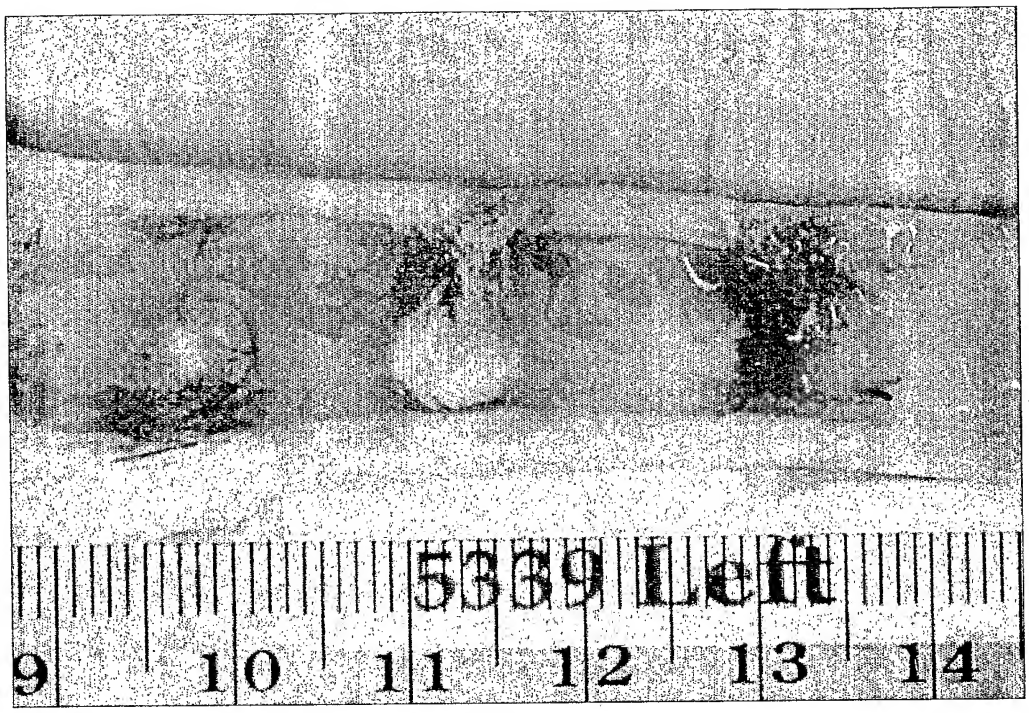

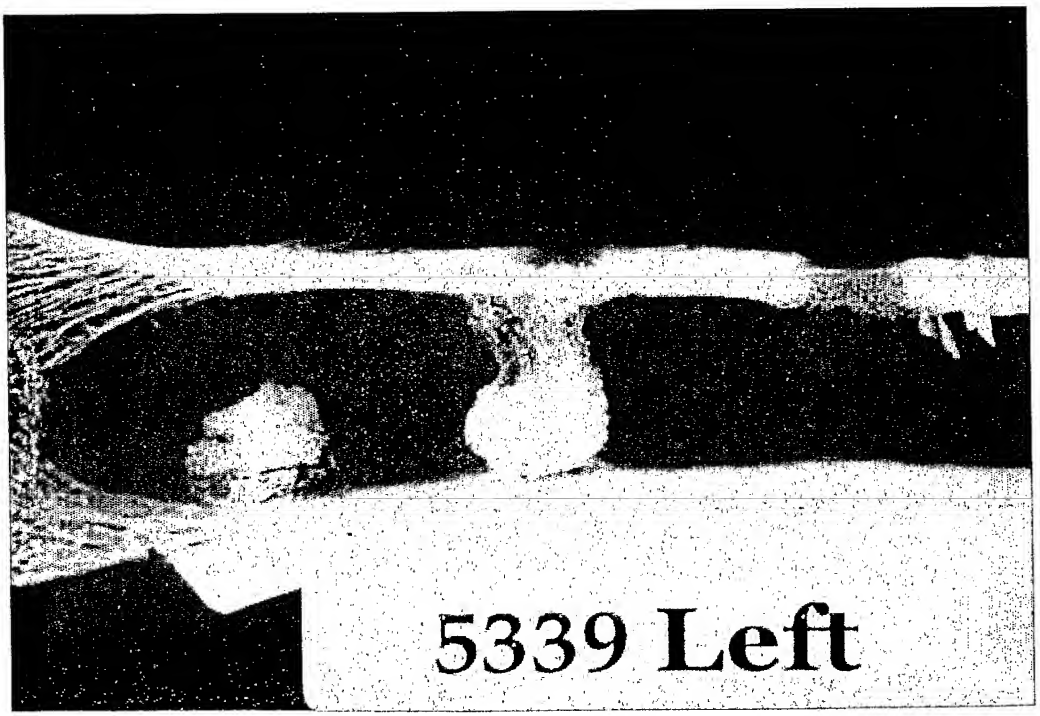

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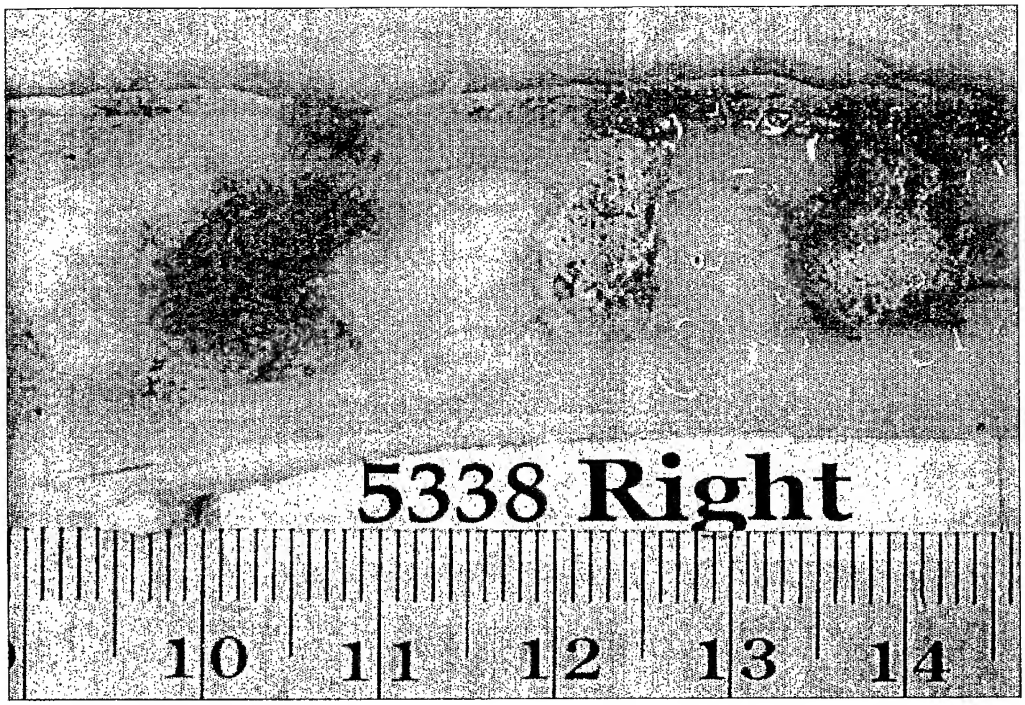

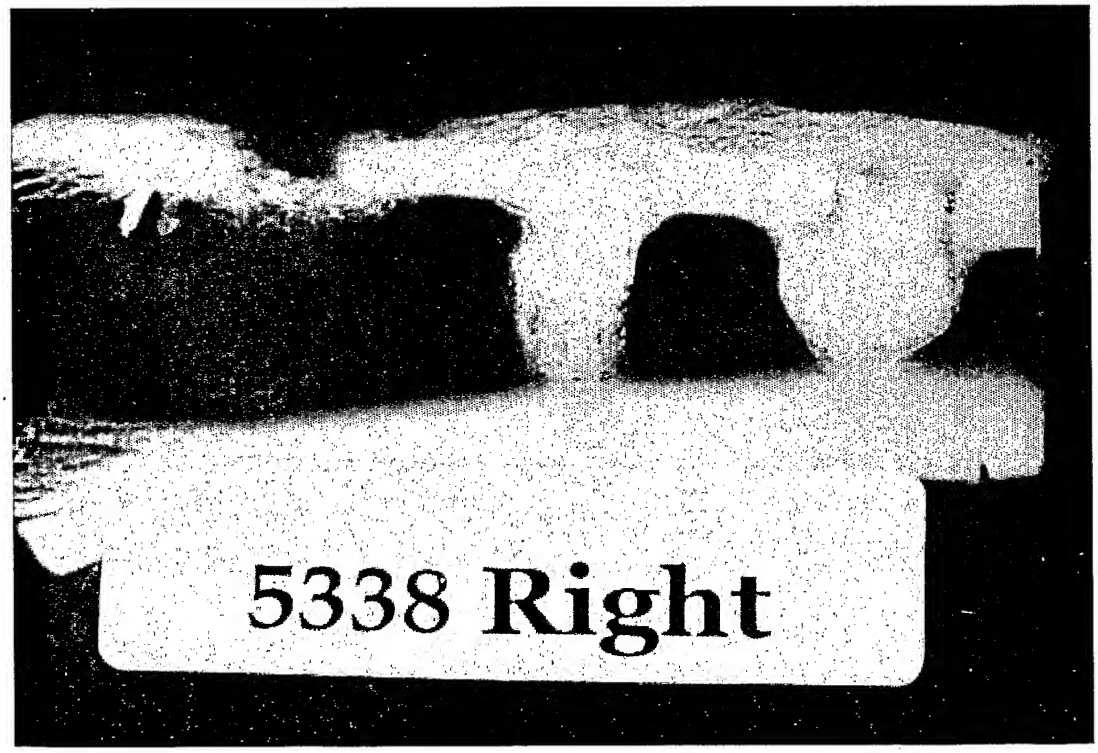

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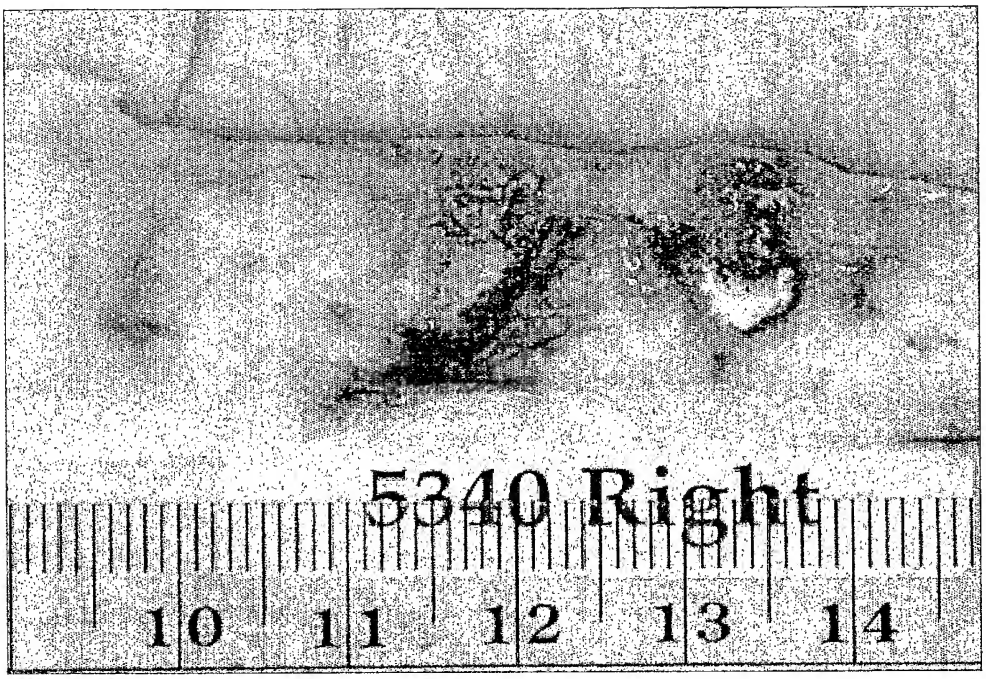

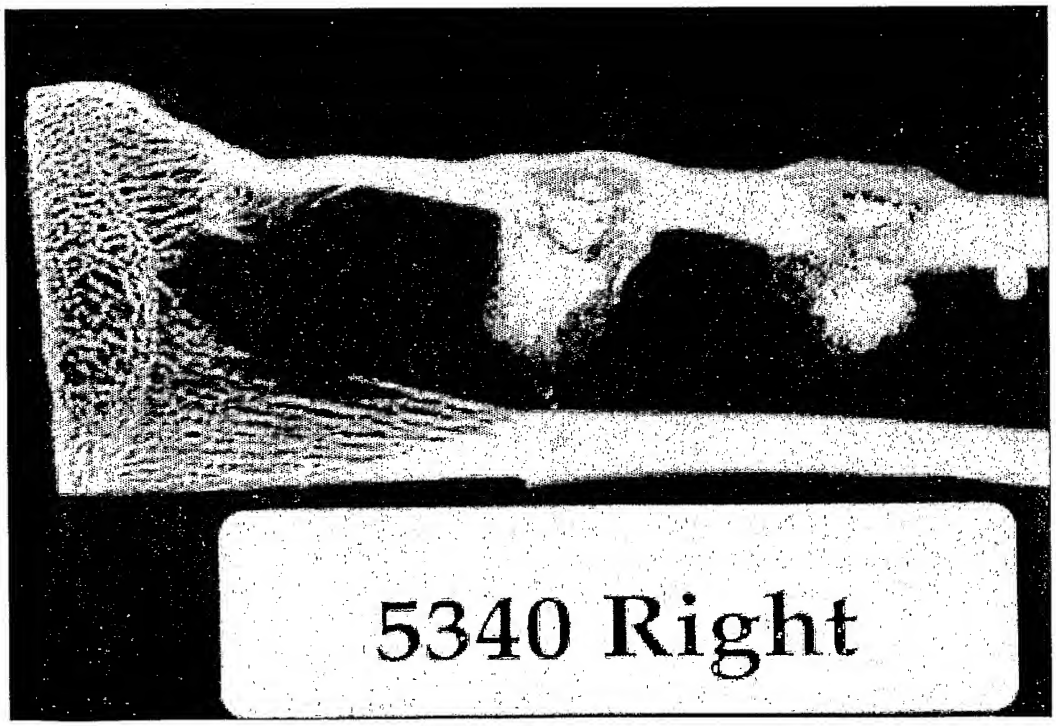

Figure 6

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<211> 431
<212> PRT
<213> Homo sapiens

<400> 1

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Met His Val Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala
  1               5                  10                  15

Leu Trp Ala Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser
             20                  25                  30

Leu Asp Asn Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser
         35                  40                  45

Gln Glu Arg Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu
     50                  55                  60

Pro His Arg Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro
 65                  70                  75                  80

Met Phe Met Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly
                 85                  90                  95

Gly Pro Gly Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser
            100                 105                 110

Thr Gln Gly Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr
        115                 120                 125

Asp Ala Asp Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys
    130                 135                 140

Glu Phe Phe His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu
145                 150                 155                 160

Ser Lys Ile Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile
                165                 170                 175

Tyr Lys Asp Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile
            180                 185                 190

Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu
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195 200 205

Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu
210 215 220

Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg
225 230 235 240

His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser
245 250 255

Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn
260 265 270

Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe
275 280 285

Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser
290 295 300

Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu
305 310 315 320

Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr
325 330 335

Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu
340 345 350

Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn
355 360 365

Ser Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His
370 375 380

Phe Ile Asn Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln
385 390 395 400

Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile
405 410 415

Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His
420 425 430

<210> 2
<211> 96
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic amino acid sequence

<400> 2
Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asp Asp Trp Ile Val Ala
1 5 10 15

Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Glu Cys Pro Phe Pro
20 25 30

Leu Ala Asp His Phe Asn Ser Thr Asn His Ala Val Val Gln Thr Leu

35 40 45

Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val Pro Thr
50 55 60

Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu Lys Val
65 70 75 80

Val Leu Lys Tyr Asn Gln Glu Met Val Val Glu Gly Cys Gly Cys Arg
85 90 95

<210> 3
<211> 96
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic amino acid sequence

<400> 3
Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val Ala
1 5 10 15

Pro Pro Gly Tyr His Ala Phe Tyr Cys His Gly Glu Cys Pro Phe Pro
20 25 30

Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Val Val Gln Thr Leu
35 40 45

Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val Pro Thr
50 55 60

Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu Lys Val
65 70 75 80

Val Leu Lys Tyr Asn Gln Glu Met Val Val Glu Gly Cys Gly Cys Arg
85 90 95

<210> 4
<211> 102
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: OPX

<220>
<221> MOD_RES
<222> (1)..(102)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the specification

<400> 4
Cys Xaa Xaa His Glu Leu Tyr Val Xaa Phe Xaa Asp Leu Gly Trp Xaa
1 5 10 15

Asp Trp Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly
20 25 30

```
Glu Cys Xaa Phe Pro Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala
          35                  40                  45

Ile Xaa Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa Xaa Val Pro Lys
      50                  55                  60

Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa
 65                  70                  75                  80

Asp Xaa Ser Xaa Asn Val Xaa Leu Xaa Lys Xaa Arg Asn Met Val Val
                 85                  90                  95

Xaa Ala Cys Gly Cys His
            100
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<210> 5
<211> 97
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Generic Sequence 7

<220>
<221> MOD_RES
<222> (1)..(97)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the specification

<400> 5

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Leu Xaa Xaa Xaa Phe Xaa Xaa Xaa Gly Trp Xaa Xaa Xaa Xaa Xaa Xaa
  1               5                  10                  15

Pro Xaa Xaa Xaa Xaa Ala Xaa Tyr Cys Xaa Gly Xaa Cys Xaa Xaa Pro
            20                  25                  30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asn His Ala Xaa Xaa Xaa Xaa Xaa
          35                  40                  45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Cys Xaa Pro
      50                  55                  60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65                  70                  75                  80

Val Xaa Leu Xaa Xaa Xaa Xaa Xaa Met Xaa Val Xaa Xaa Cys Xaa Cys
                 85                  90                  95

Xaa
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<210> 6
<211> 102
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Generic Sequence 8

<220>
<221> MOD_RES
<222> (1)..(102)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the specification

<400> 6
Cys Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Phe Xaa Xaa Xaa Gly Trp Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa Xaa Ala Xaa Tyr Cys Xaa Gly
20 25 30

Xaa Cys Xaa Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asn His Ala
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Cys Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa
65 70 75 80

Xaa Xaa Xaa Xaa Xaa Val Xaa Leu Xaa Xaa Xaa Xaa Xaa Met Xaa Val
85 90 95

Xaa Xaa Cys Xaa Cys Xaa
100

<210> 7
<211> 97
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Generic Sequence 9

<220>
<221> MOD_RES
<222> (1)..(97)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the specification

<400> 7
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly Xaa Cys Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Pro
50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65 70 75 80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys
85 90 95

Xaa

<210> 8
<211> 102
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Generic Sequence 10

<220>
<221> MOD_RES
<222> (1)..(102)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the specification

<400> 8
Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly
20 25 30

Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Xaa Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa
65 70 75 80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
85 90 95

Xaa Xaa Cys Xaa Cys Xaa
100

<210> 9
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Consensus sequence

<220>
<221> MOD_RES
<222> (2)..(5)
<223> Xaa is independently selected from a group of one or more specified amino acids as defined in the

specification

<400> 9
Cys Xaa Xaa Xaa Xaa
1 5

<210> 10
<211> 1822
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (49)..(1341)

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Met His Val
1

cgc tca ctg cga gct gcg gcg ccg cac agc ttc gtg gcg ctc tgg gca 105
Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala
5 10 15

ccc ctg ttc ctg ctg cgc tcc gcc ctg gcc gac ttc agc ctg gac aac 153
Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn
20 25 30 35

gag gtg cac tcg agc ttc atc cac cgg cgc ctc cgc agc cag gag cgg 201
Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg
40 45 50

cgg gag atg cag cgc gag atc ctc tcc att ttg ggc ttg ccc cac cgc 249
Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg
55 60 65

ccg cgc ccg cac ctc cag ggc aag cac aac tcg gca ccc atg ttc atg 297
Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met
70 75 80

ctg gac ctg tac aac gcc atg gcg gtg gag gag ggc ggc ggg ccc ggc 345
Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly Gly Pro Gly
85 90 95

ggc cag ggc ttc tcc tac ccc tac aag gcc gtc ttc agt acc cag ggc 393
Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr Gln Gly
100 105 110 115

ccc cct ctg gcc agc ctg caa gat agc cat ttc ctc acc gac gcc gac 441
Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala Asp
120 125 130

atg gtc atg agc ttc gtc aac ctc gtg gaa cat gac aag gaa ttc ttc 489
Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu Phe Phe
135 140 145

cac cca cgc tac cac cat cga gag ttc cgg ttt gat ctt tcc aag atc 537
His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile
150 155 160

cca gaa ggg gaa gct gtc acg gca gcc gaa ttc cgg atc tac aag gac 585

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Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Asp
    165                 170                 175

tac atc cgg gaa cgc ttc gac aat gag acg ttc cgg atc agc gtt tat   633
Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile Ser Val Tyr
180                 185                 190                 195

cag gtg ctc cag gag cac ttg ggc agg gaa tcg gat ctc ttc ctg ctc   681
Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu Phe Leu Leu
                200                 205                 210

gac agc cgt acc ctc tgg gcc tcg gag gag ggc tgg ctg gtg ttt gac   729
Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu Val Phe Asp
            215                 220                 225

atc aca gcc acc agc aac cac tgg gtg gtc aat ccg cgg cac aac ctg   777
Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg His Asn Leu
        230                 235                 240

ggc ctg cag ctc tcg gtg gag acg ctg gat ggg cag agc atc aac ccc   825
Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile Asn Pro
    245                 250                 255

aag ttg gcg ggc ctg att ggg cgg cac ggg ccc cag aac aag cag ccc   873
Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys Gln Pro
260                 265                 270                 275

ttc atg gtg gct ttc ttc aag gcc acg gag gtc cac ttc cgc agc atc   921
Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe Arg Ser Ile
                280                 285                 290

cgg tcc acg ggg agc aaa cag cgc agc cag aac cgc tcc aag acg ccc   969
Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro
            295                 300                 305

aag aac cag gaa gcc ctg cgg atg gcc aac gtg gca gag aac agc agc   1017
Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser
        310                 315                 320

agc gac cag agg cag gcc tgt aag aag cac gag ctg tat gtc agc ttc   1065
Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe
    325                 330                 335

cga gac ctg ggc tgg cag gac tgg atc atc gcg cct gaa ggc tac gcc   1113
Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala
340                 345                 350                 355

gcc tac tac tgt gag ggg gag tgt gcc ttc cct ctg aac tcc tac atg   1161
Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met
                360                 365                 370
aac gcc acc aac cac gcc atc gtg cag acg ctg gtc cac ttc atc aac   1209
Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn
            375                 380                 385

ccg gaa acg gtg ccc aag ccc tgc tgt gcg ccc acg cag ctc aat gcc   1257
Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala
        390                 395                 400

atc tcc gtc ctc tac ttc gat gac agc tcc aac gtc atc ctg aag aaa   1305
Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys
    405                 410                 415
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tac aga aac atg gtg gtc cgg gcc tgt ggc tgc cac tagctcctcc        1351
Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His
420                 425                 430

gagaattcag acccttttggg gccaagtttt tctggatcct ccattgctcg ccttggccag 1411

gaaccagcag accaactgcc ttttgtgaga ccttcccctc cctatcccca actttaaagg 1471

tgtgagagta ttaggaaaca tgagcagcat atggcttttg atcagttttt cagtggcagc 1531

atccaatgaa caagatccta caagctgtgc aggcaaaacc tagcaggaaa aaaaaacaac 1591

gcataaagaa aaatggccgg gccaggtcat tggctgggaa gtctcagcca tgcacggact 1651

cgtttccaga ggtaattatg agcgcctacc agccaggcca cccagccgtg ggaggaaggg 1711

ggcgtggcaa ggggtgggca cattggtgtc tgtgcgaaag gaaaattgac ccggaagttc 1771

ctgtaataaa tgtcacaata aaacgaatga atgaaaaaaa aaaaaaaaaa a          1822
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